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14. ABSTRACT
Loss of TSC pathway components alters the timing of neuronal differentiation in the Drosophila eye and wing imaginal disc. To determine the mechanisms underlying this regulation of neuronal differentiation, we have 1) further defined the subtypes of photoreceptors that respond to loss of TSC, 2) tested candidates for the regulation of differentiation in the eye, Our data indicate that loss of TSC does not control neural differentiation through 5' TOP elements contained in the mRNA of proneural genes examined (CG11799, echinoid, moleskin, src). Consistent with this conclusion, our genetic analysis of the Drosophila homolog of polypyrimidine tract binding protein, Hephaestus, indicates that loss of Hephaestus does not alter the timing of differentiation of photoreceptors in the eye. We conclude therefore that alternate mechanisms control this process. Our epistasis analysis indicates that S6K is essential for the precocious differentiation seen in TSC clones, while loss of eIF4E does not affect the timing of differentiation. The Ecdysone receptor (Ecr) pathway also regulates the timing of differentiation in the eye. Our genetic analysis indicates that EcR functions in a parallel pathway to TSC in the timing of differentiation.

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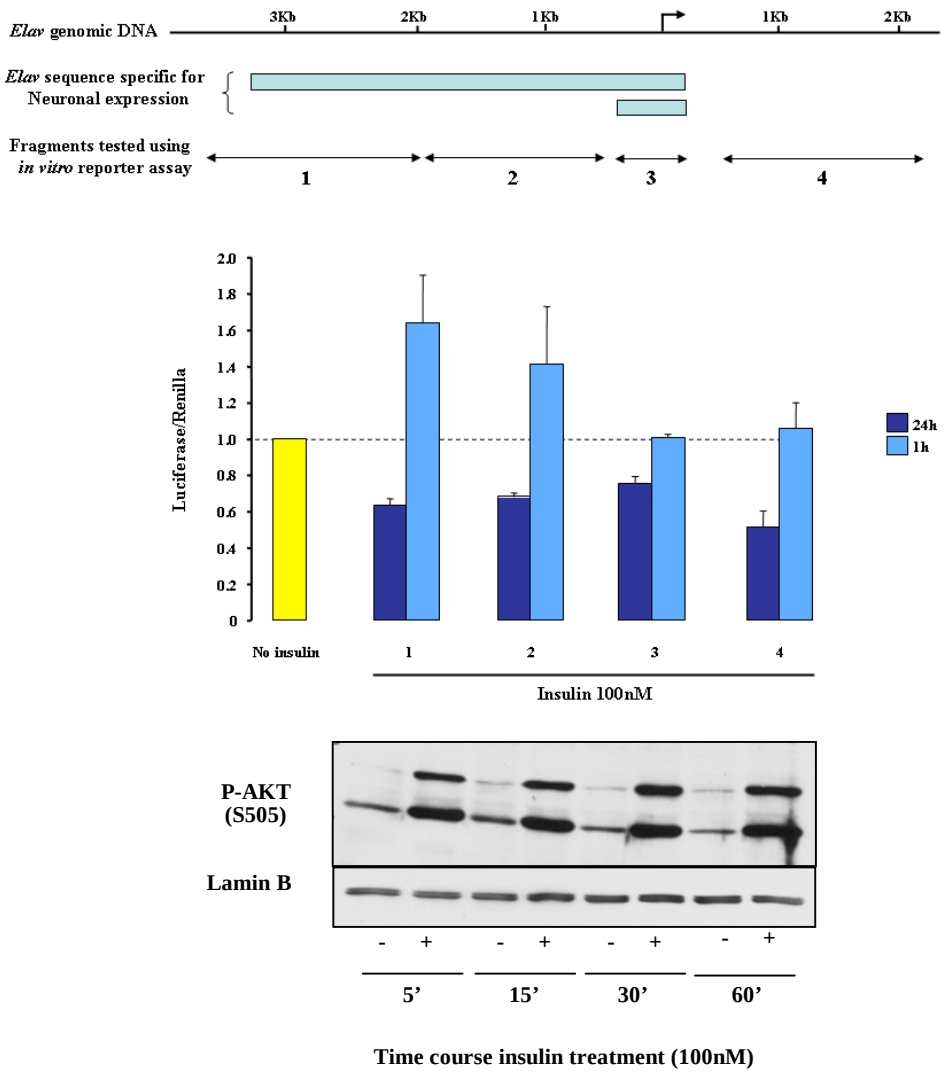
INTRODUCTION: Tuberous Sclerosis complex (TSC) is one of the most common forms of neurocutaneous disorders, affecting 1 in 6000 live births. This autosomal dominant disease typically presents in the pediatric age group with neuropsychiatric signs and symptoms of epilepsy, mental retardation and autism. It is poorly understood how loss of TSC leads to these neurological defects. We have shown that TSC plays a key role in controlling the timing of neuronal differentiation in *Drosophila* through the conserved insulin receptor (InR)/Tor kinase signalling pathway (Bateman &McNeill, 2004). The goal of our current research is to determine the mechanism by which TSC regulates the timing of neuronal differentiation. To uncover the pathway(s) downstream of TSC in the temporal control of neuronal differentiation we have taken complementary approaches to address both the transcriptional and translational outputs of the TSC pathway. *Task 1.* Identify minimal region of neuronal promoters responsive to TSC. *Task 2 & Task 3.* Test candidates for control of neural differentiation using an RNAi screen of the *Drosophila* genome.

BODY: **Task 1) Analysis of minimal regions of neuronal promoters responsive to TSC.** Our preliminary data indicated that loss of TSC leads to increased expression of PntP2 transcript, as assessed by a PntP2 specific enhancer trap, and increased expression of the neural specific splicing factor ELAV (as detailed in the grant application). During year 1 of this proposal, we verified that PntP2 expression increases with RT-PCR (as detailed in the first progress report). To determine the enhancer elements responsible for the increase in PntP2 transcription upon loss of TSC, we made enhancer-reporter constructs, and generated transgenic fly lines carrying the upstream elements of the PntP2 genomic region. Preliminary analysis of these lines indicated that a 4kb element can confer PntP2 expression in the eye imaginal disc. In parallel, we determined that overexpressing PntP2 is not sufficient to drive precocious neuronal differentiation, suggesting other components downstream of TSC are needed for precocious differentiation (McNeill et al, 2008; ***Genetics: see Appendix 1***).

During the second year, we therefore focused our attention on examining the enhancer region of the Elav promoter for TSC-responsive elements, since ELAV responds to loss of TSC, and loss and gain of function analysis indicates that ELAV is essential for proper neural differentiation. We also found that loss of TSC leads to phosphorylation of PntP2, providing a potential mechanism to complement the increase of PntP2 in the regulation of neuronal differentiation.

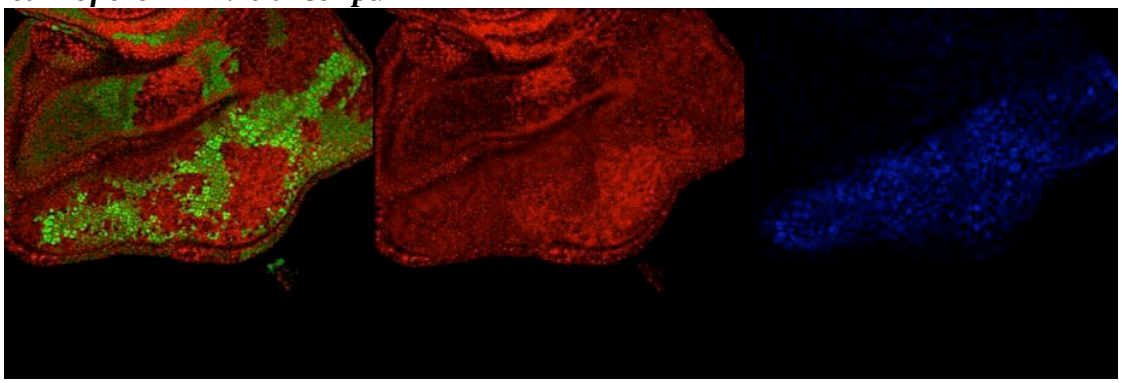
We initiated these studies by cloning fragments of the upstream genomic region of ELAV, to identify potential TSC responsive elements. In our initial studies, we cloned these fragments upstream of a luciferase reporter, transfected these constructs into S2 *Drosophila* cells, and tested if these fragments conferred responsiveness to activation of the Insulin Receptor/TSC pathway by addition of insulin. Two days after transfection, cells were treated with stimulating concentration of insulin for 1h and 24h. These studies revealed that insulin treatment induces a transient increase of luciferase activity for Elav promoter fragment #1 and #2 (upper panel). As a readout to verify the activation of the InR pathway in response to insulin treatment, we also assessed AKT phosphorylation using western blot analysis (lower panel). Lamin B was used as a loading control.

Figure 1. **Fragments of the *Elav* promoter respond to insulin treatment in S2 cells**

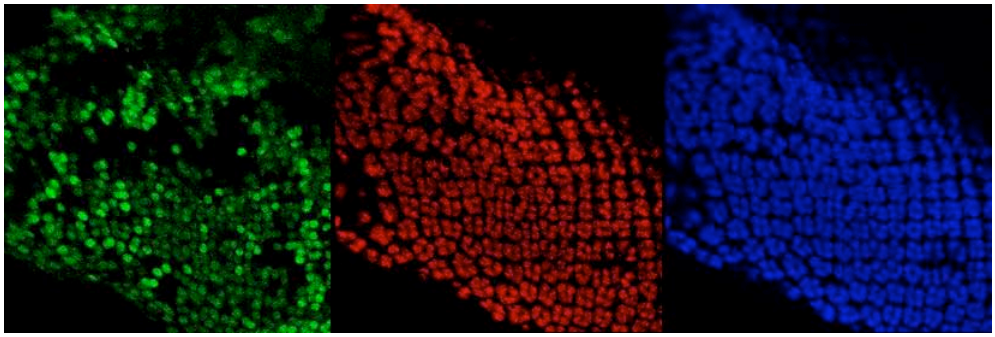


To determine if the response of these fragments to Insulin treatment is physiologically relevant, and if they respond to loss of TSC *in vivo*, we examined the responsivity of these enhancer elements in transgenic *Drosophila* analysis, We generated transgenic flies with different two reporter constructs where expression of Beta-galactosidase was placed under the control of the 3.5Kbp and the 390bp *Elav* promoter. Loss of function clones of TSC1 were generated in each background, and expression of the reporter was assessed by staining with antibodies to beta-galactosidase. We determined that *TSC1* LOF clones induce an increase in reporter activity when under the control of the 3.5Kb *Elav* promoter fragment, but that *TSC1* LOF clones do not change activity of the reporter when under the control of the 390bp *Elav* promoter fragment (Figure 2). These data indicate that a portion of the Elav promoter from -3.0kb to -.4 kb confer responsiveness to TSC *in vivo*.

Figure 2 . ***TSC controls expression of ELAV transcription via an element from -3kb to -.4 kb upstream of the ELAV transcript.***



GFP/X-Gal driven by the 3.5kb element /Elav



GFP/X-Gal driven by 390 bp element/Elav

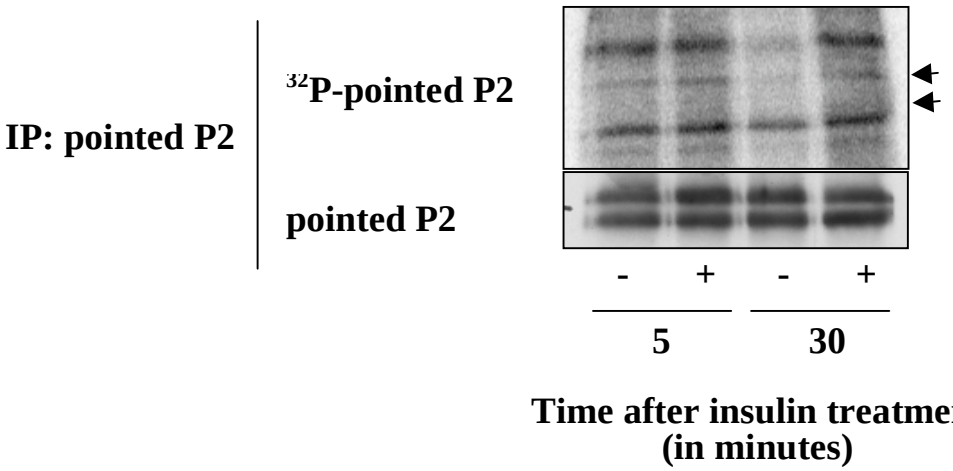
X-Gal expression is increased in *TSC1* LOF clones (marked by the absence of GFP), suggesting that it includes an element responsive to InR/TSC signalling (upper panel). However *TSC1* LOF clones do not alter the expression of X-Gal when driven by the 390bp *Elav* promoter fragment (lower panel).

Regulation of pointed P2 phosphorylation by the InR/TSC pathway

We have previously shown that the InR/TSC pathway specifically regulates the expression levels of the ETS transcription factor pointed P2 (Bateman and McNeill, 2004), and that reduction of pointed P2 expression phenocopies InR/TSC mutants in the regulation of differentiation but not growth (McNeill et al, 2008). Pointed P2 function is dependent on site specific phosphorylation, thus a plausible model is that InR/TSC both increases PntP2 transcription (McNeill et al, 2008) and modulates pointed P2 activity by altering its phosphorylation status. Our preliminary data support this hypothesis

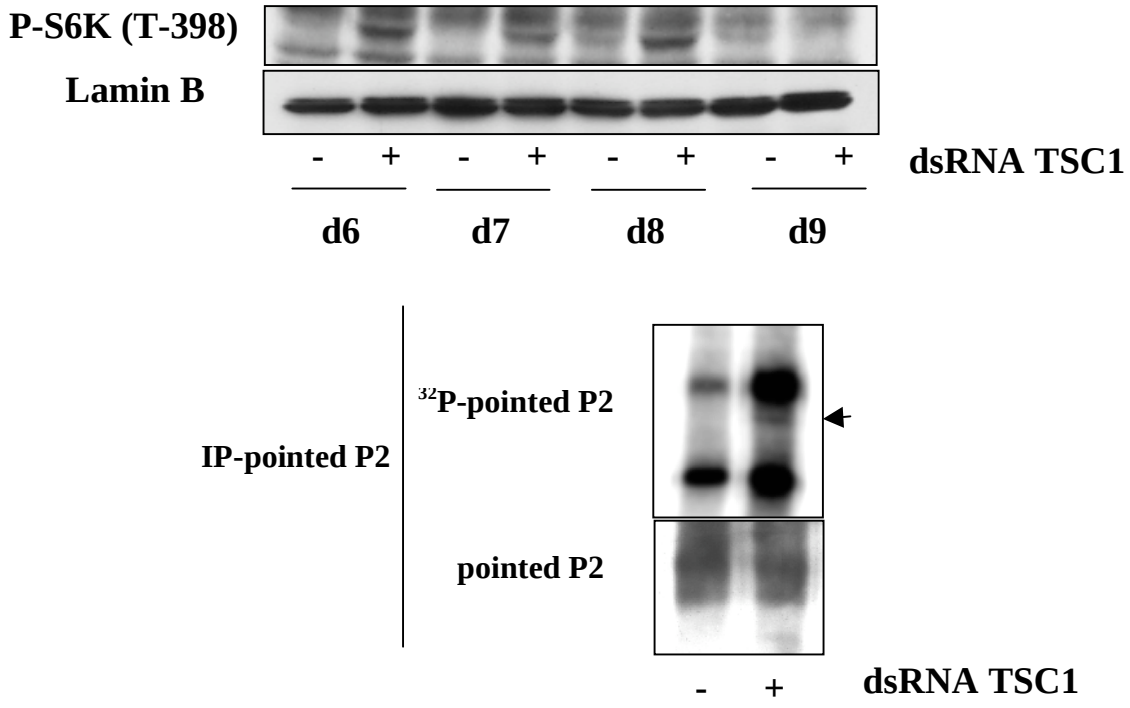
To test if PntP2 is phosphorylated by insulin signaling we conducted *in vivo* labeling experiments with ^{32}P, using S2 cells. After a prelabeling period, we stimulated cells with insulin, lysed cells and immunoprecipitated (IP) with antibodies we generated that were specific to PntP2. SDS-PAGE analysis of the immunoprecipitate, followed by autoradiograph analysis, showed an increase of endogenous phosphorylated-pointed P2 after 30 minutes of treatment with insulin.

Figure 3. Insulin treatment increases pointed P2 phosphorylation in S2 cells.



To determine if PntP2 phosphorylation is specifically increased by removal of TSC, we used a similar labeling protocol, and exposed S2 cells to dsRNA to remove TSC1. We noted that exposure of cells to dsRNA to TSC1 also led to an increase in the phosphorylation of PntP2. Since it is known that phosphorylation of PntP2 is needed for its transcriptional activity, this supports a model in which TSC regulates neuronal differentiation by increasing the phosphorylation of PntP2.

Figure 4. Activation of InR/TOR pathway using TSC1 dsRNA increases pointed P2 phosphorylation



DsRNA against TSC1 efficiently activates InR/TOR signalling as illustrated by the increase of phospho-S6K after 6, 7, and 8 days in culture. (top panel). DsRNA against TSC1 induces an increase of pointed P2 phosphorylation (bottom panel).

Although the signal from the *in vivo* labelling is weak, it seems that both bands corresponding to phospho-pointed P2 are decreased in the presence of dsRNA against pointed P2 in the control experiment and in response to a 30 minutes treatment with insulin. We will optimize this by conducting a detailed time course, and using a more robust protocol in which signal for phospho-pointed P2 is enhanced.

In summary, our preliminary data showed that activation of the InR/TSC signaling, either by treating with insulin, or in response to dsRNA against TSC1 increases pointed P2 phosphorylation. To determine if the phosphorylation site on PntP2 that is targeted by TSC is the same site that has been previously been described to be the target of MAPK signaling tested PntP2 which is mutated at this site, and used it to determine if the increase in phosphorylation seen upon insulin stimulation is blocked by this mutation. We found that the increase occurred even in the case of

mutated PntP2, indicating that other sites, outside the canonical MAPK target site are phosphorylated when S2 cells are stimulated with Insulin.

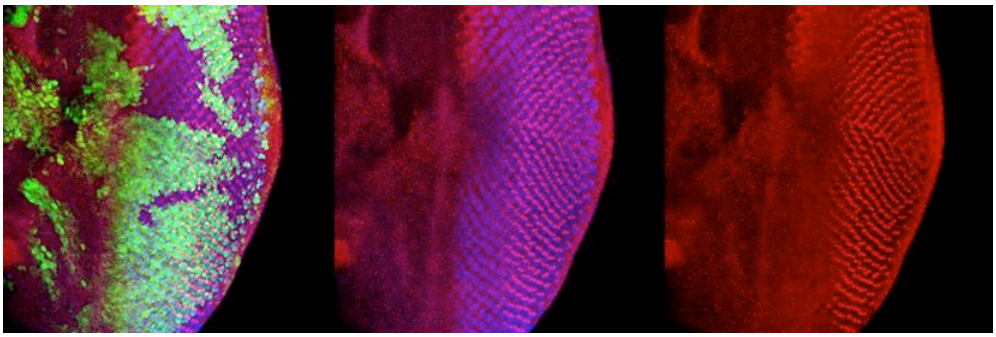
Task 2 and Task 3. Analysis of candidates for TSC -mediated control of neuronal differentiation using genome-wide RNAi screens and genetic epistasis analysis.

We have previously shown that loss of TSC in the *Drosophila* eye leads to precocious neural differentiation, without altering the specific cell fate decisions (Bateman and McNeill, 2004). Regulation of growth by the InR/Tor signaling pathway is mediated in part through translational control. In the first year of this grant, as detailed in the previous report, we tested candidates for translational control via an in silico screen of 5'TOP containing genes in a genome-wide analysis. No neuronal proteins that are translated from transcripts with 5'TOPs (including Mnf, ed, dim7, Src42A and Src64B) showed any changes in levels of translation in the absence of tsc or pten, negative regulators of the InR/Tor pathway. Loss of Heph, a 5'TOP binding protein, did not alter the timing of differentiation. Our results suggested that 5'TOP-containing neuronal transcripts are not the link between the InR/Tor growth pathway and the Egfr differentiation pathway. Therefore we continued our analysis of potential control by examining other mechanisms. During the course of the first year of this grant, a study was reported that combined the use of rapamycin, transcriptional profiling, and RNA interference in *Drosophila* tissue culture cells, and identified a set of Tor-regulated genes that control growth (Guertin et al., 2006). As TOR lies downstream of TSC, these genes provide a set of validated targets that have the potential to regulate the timing of neuronal differentiation under the control of the InR/TSC pathway.

In years two and three of this grant, we conducted an *in vivo* RNAi screen to determine if these genes regulated by rapamycin in tissue culture are involved in controlling the timing of photoreceptor differentiation in vivo. These studies took advantage of a new resource, a genome-wide RNAi transgenic *Drosophila* collection (Dietzl et al, 2007), maintained by the VDRC (http://www.vdrc.at/rnai-library). The library comprises 22,247 transgenic *Drosophila* strains, each containing an inducible UAS-RNAi construct against a single protein coding gene. 12,251 genes, or 88.2% of the Drosophila genome, are represented in this collection. All insertions have been molecularly validated, and a sample also functionally validated. By crossing lines carrying a specific dsRNA to Gal4 driver lines we can determine if genes regulated by the InR/TSC pathway control the timing of differentiation. We obtained inducible RNAi lines to the candidates, and

tested their ability to alter the timing of differentiation. We have examined thus far examined 25 lines, of which 22 show no effect on the timing of differentiation (for example, see Figure 5). However some lines, specifically CG1242 (Figure 6) , CG1201(Figure 7) , CG4260, and CG6603 do show altered timing of differentiation .

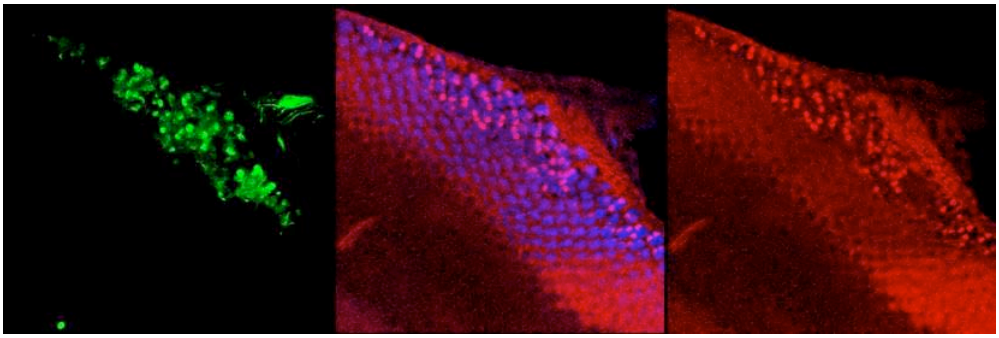
Figure 5. Clones of cells expressing dsRNA for CG6677 (Ash2) do not change the timing of photoreceptor differentiation



GFP/Bar/Elav

RNAi clones for CG6677 (marked by GFP) do not show any alteration in the timing of differentiation of photoreceptor stained by Bar

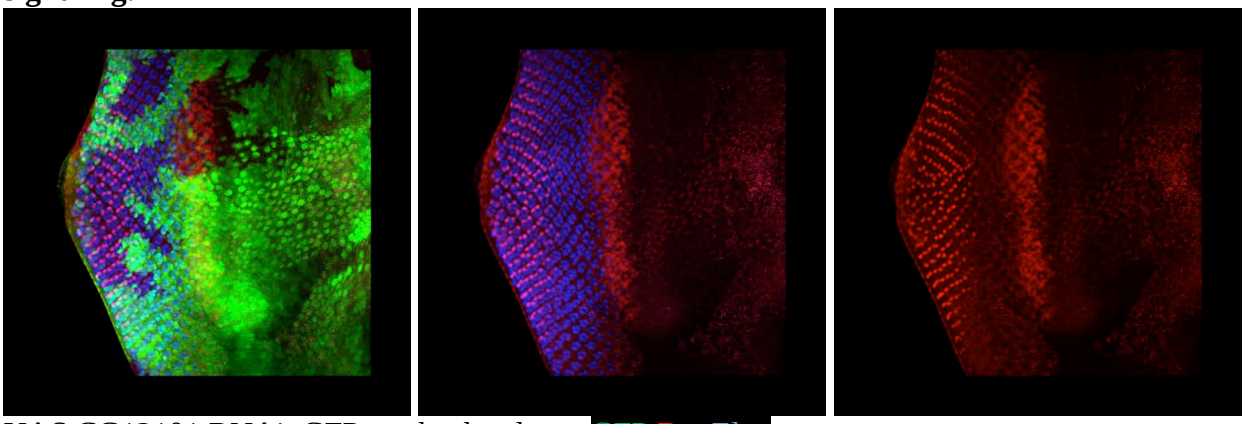
Figure 6. RNAi clones for CG1242 delay the timing of photoreceptor differentiation



GFP/Bar/Elav

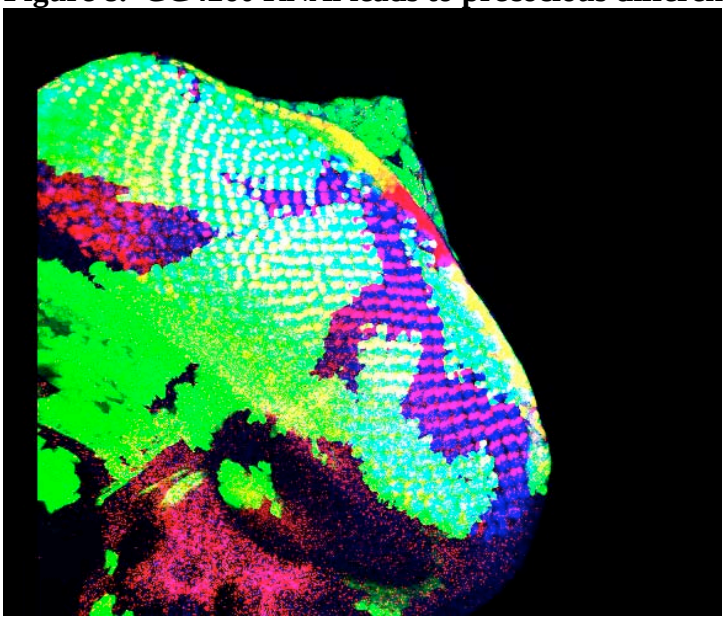
RNAi clones for CG1242 (marked by GFP) show delay in the expression of Bar in PRs, while Elav does not seem to be altered. We could not make clones at a later stage during eye development possibly because of an effect of sustained downregulation of CG1242 on cell viability.

Figure 7. CG12101 RNAi delays differentiation of R 1 and 6, similar to loss of InR/TOR signaling.



UAS CG12101 RNAi, GFP marks the clones GFP Bar Elav
This phenotype is similar to loss of InR/Tor signaling.

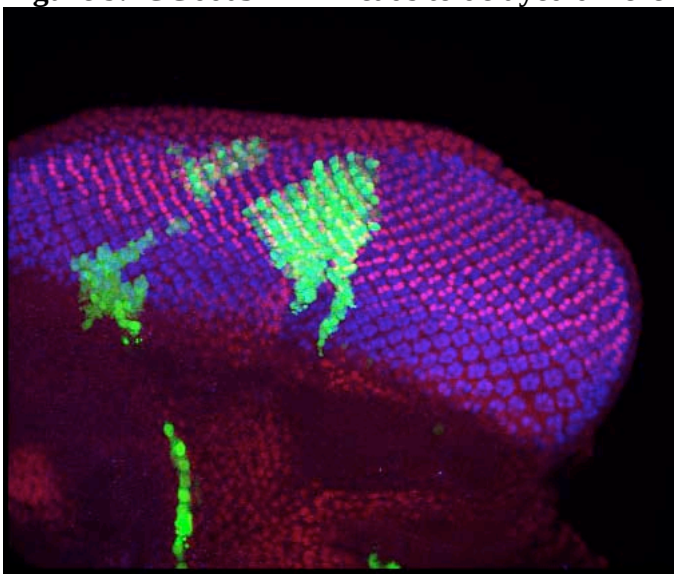
Figure 8. CG4260 RNAi leads to precocious differentiation



Clones of cells expressing UAS-CG4260 RNAi, marked by expression of GFP(Green) were stained with antibodies to Bar (Red), as well as ELAV (Blue). Knockdown of CG4260 leads to precocious expression of Bar, phenocopying loss of TSC1.

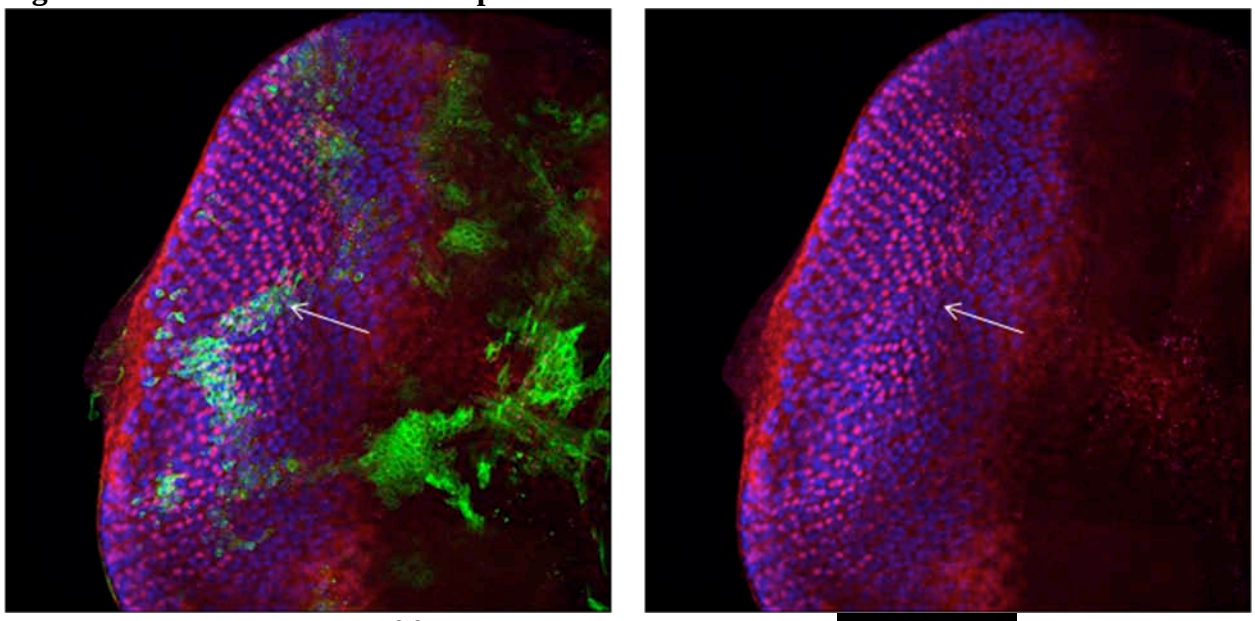
These data show that CG4260, CG6603 and CG12101, which are altered by rapamycin in tissue cuture models, phenocopy alterations in TSC/TOR signaling in the timing of differentiation. To test genetically if loss of these genes function downstream of TSC/TOR signaling, we generated clones of cells doubly mutant for known InR/Tor pathway components and expressing either CG4260, CG6603 or CG12101 RNAi (Figure 10, Figure 11 and data not shown).

Figure 9. CG6603 RNAi leads to delayed differentiation



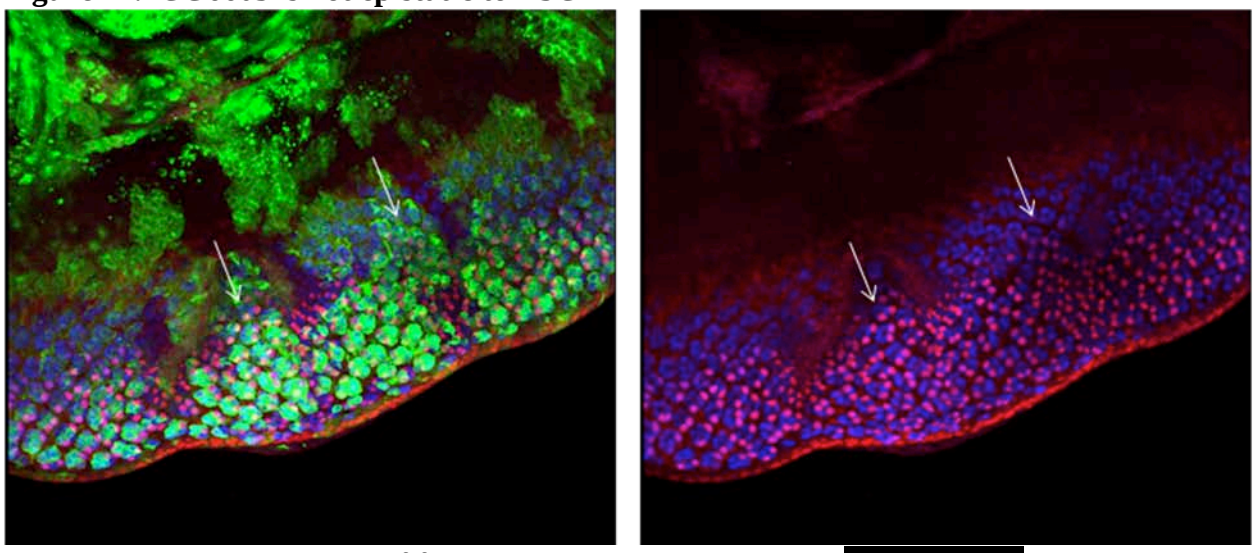
Loss of CG6603 by expression of RNAi (marked by green) delays expression of Bar (red) in photoreceptors R1 and R6, as seen with loss of InR/Tor signaling

Figure 10. CG4260 RNAi is not epistatic to Rheb loss of function



UAS 4260 RNAi; Rheb loss of function, GFP marks the clones. GFP Bar Elav

Figure 11. CG6603 is not epistatic to TSC1



UAS 6603 RNAi; TSC1 loss of function, GFP marks the clones. GFP Bar Elav

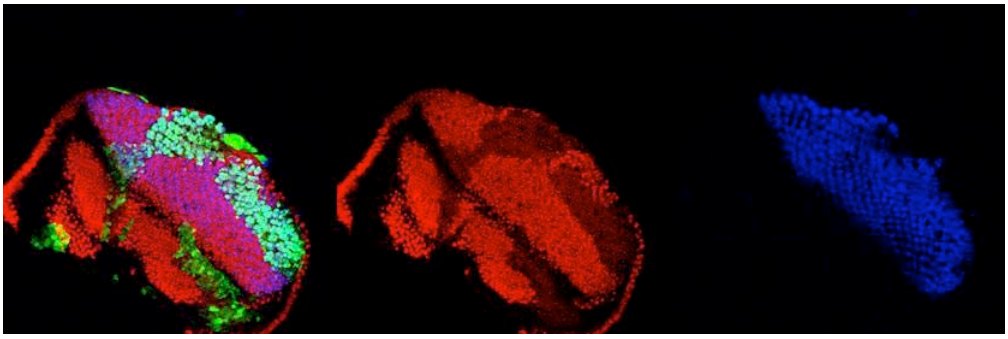
Thus while loss of CG6603, CG4260 and CG12101 phenocopy disruptions in the TSC pathway, genetic analysis indicates that TSC1 is epistatic to these genes in the control of the timing of differentiation. These data indicate that if CG6603, CG12101 and CG4260 do indeed act downstream of TSC1 in the control of the timing of differentiation, they cannot be solely responsible for this. A reasonable hypothesis is that these genes function together downstream of TSC1. To test if these identified mediators function together, we will need to remove both at the same time in clones of cells that also lack TSC1. These studies are genetically cumbersome, and to complete them is outside the scope of the current grant. We hope to obtain additional funding to extend these studies.

To better understand the signal transduction system downstream of TSC in neuronal differentiation, we also continued our genetic epistasis analysis of known components of the pathway in the eye imaginal disc. We found that loss of eIF4E does not affect the timing of differentiation, suggesting it is not a key element of the differentiation control. Strikingly, while loss of S6 has little effect on its own, double mutant clones of S6 and TSC leads to an abrogation of precocious differentiation, suggesting that S6 activation is crucial to the promotion of differentiation. This work is detailed in McNeill et al., (2008): see Appendix 1. We also found that loss of FOXO lead to dramatic changes in the timing of differentiation, as does loss of Raptor.

Finally, we have also tested elements of the nuclear receptor ecdysone pathway, which is also known to regulate the timing of differentiation in the eye, to determine the genetic relationship between these two pathways.

Ecdysone signalling acts as a developmental timer to coordinate tissue differentiation. It controls the progression of the morphogenetic furrow during eye development (Brennan et al, 1998: Figure 12) suggesting that, similarly to InR/TSC signalling, ecdysone regulates the timing of photoreceptor differentiation. Determining the relationship between both pathways may provide us with a mechanism to explain how InR/TSC regulates neuronal differentiation.

Figure 12. The ecdysone receptor (EcR) regulates the timing of photoreceptor differentiation



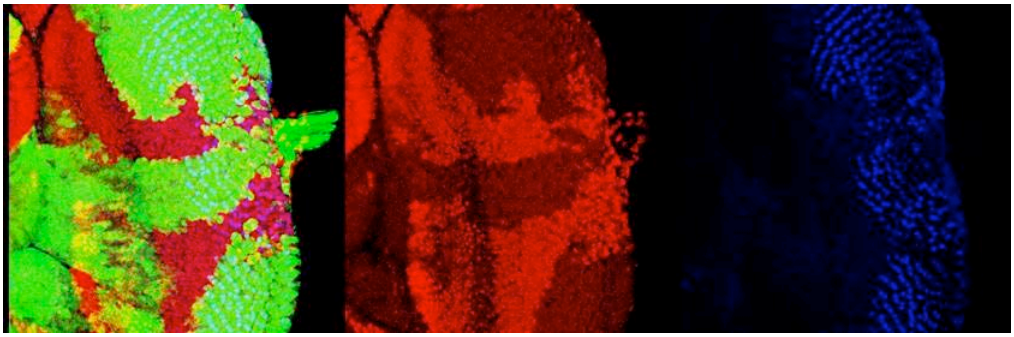
GFP/EcR/Elav

RNAi clones for EcR (marked by GFP) show efficient downregulation of EcR, and acceleration of photoreceptor differentiation as illustrated by Elav staining.

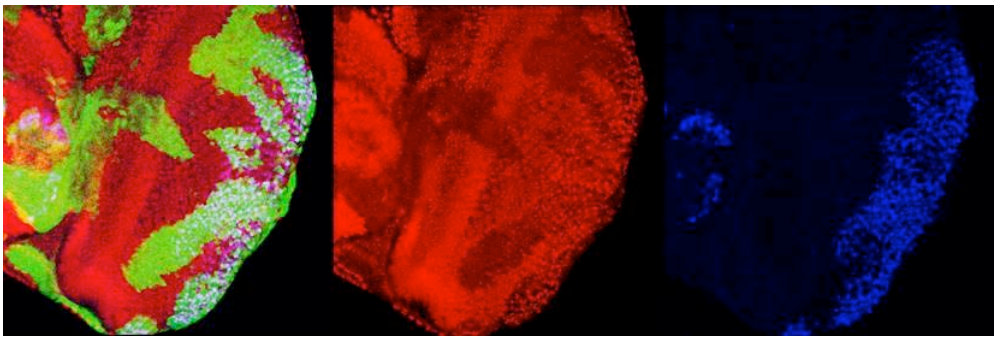
To determine if the EcR also regulates the timing of neuronal differentiation in other tissues besides the eye primordium, we have examined the effect of loss of function clones of TSC1 and RNAi to EcR in the leg disc and the antennal disc (Figure 9). These studies revealed that EcR and InR/TSC similarly regulate the timing of expression of the neuronal marker Elav in antennal discs.

To determine the epistatic relationship between the InR/TSC pathway and the EcR pathway, we generated clones doubly mutant for both pathways. We used mitotic recombination to generate clones of cells that are mutant for the *InR*, and which simultaneously express dsRNAi for EcR. These clones do not show expression of Elav even when the clone crosses a cluster of Elav-expressing cells. This suggests that, as for photoreceptors, EcR acts either upstream or in parallel to InR to control the timing of neuronal differentiation in the antennal disc.

Figure 13. Temporal control of neuronal differentiation is similarly regulated by EcR and InR-TSC pathway



GFP/EcR/Bar



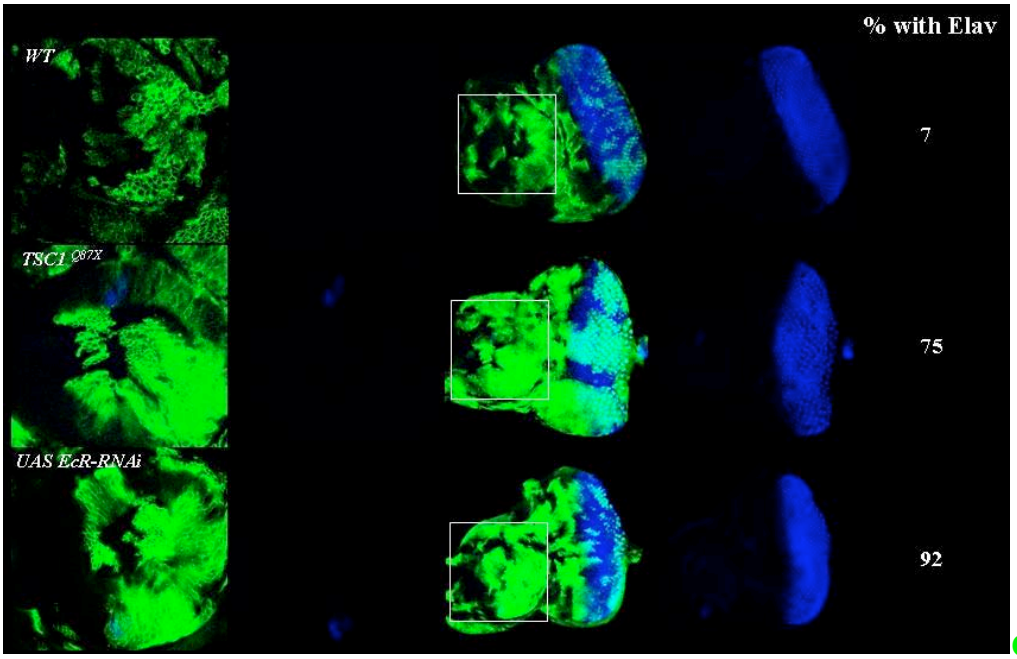
GFP/EcR/Prospero

RNAi clones for EcR (marked by GFP) show acceleration of differentiation of Bar-expressing photoreceptors (PR1 & 6) and Prospero-expressing photoreceptor R7

To complement this analysis, we also conducted epistasis experiments using mitotic recombination clones which EcR-RNAi/*InR* and examined the expression of the previously characterized EcR target, BrCZ1. We found that these clones show clear upregulation of BrCZ1 in the eye disc. This suggests that InR/TSC pathway acts either upstream or in parallel to EcR to control the expression of BrCZ1.

In addition, we generated loss- of-function clones for InR alone, or in combination with EcR RNAi. We found that there were equivalent delays in each case, consistent with a model in which EcR acts either upstream or in parallel to InR/TSC in the control of the timing of neuronal differentiation.

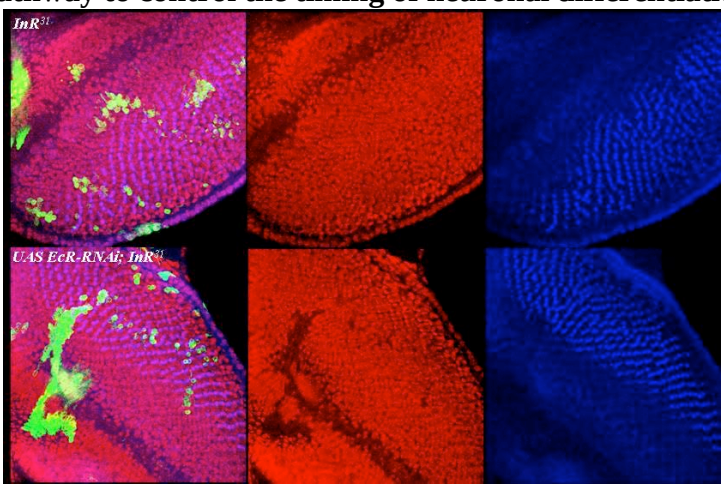
Figure 14. EcR and TSC regulate neuronal differentiation in the antennal disc.



GFP/Elav

TSC1-/- and EcR RNAi clones induce precocious expression of Elav in the antennal disc compared to the wild type. Right column displays the percentage of antennal disc stained with Elav. Advancement of the morphogenetic furrow was used to stage discs.

Figure 15. Epistasis experiments show that EcR acts either upstream or in parallel to InR-TSC pathway to control the timing of neuronal differentiation



GFP/EcR/Bar

LOF clones for *InR* alone (top panel) or in combination with RNAi against EcR (lower panel and marked by the presence of GFP) show delay in differentiation of Bar expressing cells. This suggests that EcR acts either upstream or in parallel to InR/TSC signalling in the control of the timing of PR differentiation

Summary: InR/TSC and EcR pathways similarly regulate the timing of neuronal differentiation. Epistasis experiments suggest that EcR acts in parallel to InR pathway to control this stepwise mechanism. Analysis of genes altered by rapamycin treatment in tissue culture identified novel targets of TSC in the regulation of the timing of differentiation in vivo. Genetic epistasis analysis indicates that alteration of any one of these genes is not sufficient to inhibit alteration of the TSC/TOR pathway, suggesting that TSC regulates a suite of targets to control the timing of differentiation of neural differentiation in vivo.

KEY RESEARCH ACCOMPLISHMENTS:

- PntP2 enhancer analysis and ELAV enhancer analysis in vitro and in vivo has identified regions responsive to TSC.
- Phosphorylation of PntP2 is induced by loss of TSC or activation of Insulin Receptor signaling, providing a potential dual mechanism for the regulation of the timing of differentiation.
- Genetic epistasis analysis reveals that EcR acts upstream or in parallel to InR pathway to control the timing of neuronal differentiation.
- RNAi screening of candidate genes in vivo has identified genes that are regulated by TSC that alters the timing of differentiation.

REPORTABLE OUTCOMES. A manuscript has been published, detailing the regulation of PntP2 transcription in TSC mutant clones, and the necessary but not sufficient effects of PntP2 expression (McNeill et al, 2008). A peer-reviewed literature analysis has been written on the intersection of Insulin Receptor signaling and neuronal differentiation (Bateman & McNeill, 2006)

CONCLUSION: Our studies have identified new downstream targets of the TSC pathway in photoreceptor differentiation in the *Drosophila* eye; PntP2, Broad Complex, CG. We have tested the hypothesis that 5' TOP containing transcripts and found no evidence to support this model, therefore other modes of translational control will be assessed. We have determined that while PntP2 expression is necessary for neuronal differentiation, and its expression is altered by TSC activity, overexpression of PntP2 is not sufficient to account for the effects of TSC on neuronal differentiation. We have found that PntP2 phosphorylation is enhanced by loss of TSC, suggesting that InR/TSC may regulate the timing of differentiation via control of the transcriptional activity of PntP2. We have identified novel targets of TSC in the regulation of the timing of differentiation. Future studies will determine how TSC regulates an array of genes to control differentiation in vivo.

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DOI: 10.1534/genetics.107.083097

Regulation of Neurogenesis and Epidermal Growth Factor Receptor Signaling by the Insulin Receptor/Target of Rapamycin Pathway in Drosophila

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ABSTRACT

Determining how growth and differentiation are coordinated is key to understanding normal development, as well as disease states such as cancer, where that control is lost. We have previously shown that growth and neuronal differentiation are coordinated by the insulin receptor/target of rapamycin (TOR) kinase (InR/TOR) pathway. Here we show that the control of growth and differentiation diverge downstream of TOR. TOR regulates growth by controlling the activity of S6 kinase (S6K) and eIF4E. Loss of *s6k* delays differentiation, and is epistatic to the loss of *tsc2*, indicating that S6K acts downstream or in parallel to TOR in differentiation as in growth. However, loss of *eIF4E* inhibits growth but does not affect the timing of differentiation. We also show, for the first time in Drosophila, that there is crosstalk between the InR/TOR pathway and epidermal growth factor receptor (EGFR) signaling. InR/TOR signaling regulates the expression of several EGFR pathway components including *pointedP2* (*pntP2*). In addition, reduction of EGFR signaling levels phenocopies inhibition of the InR/TOR pathway in the regulation of differentiation. Together these data suggest that InR/TOR signaling regulates the timing of differentiation through modulation of EGFR target genes in developing photoreceptors.

A fundamental challenge during the development of any complex organism is the coordination of proliferation and differentiation. The rate of proliferation is not constant during development (Neufeld *et al.* 1998) and depends on the developmental stage as well as hormonal and nutritional cues (Britton *et al.* 2002). Coordinating growth and differentiation is a particular challenge in complex tissues, such as the nervous system. Neurogenesis is preceded by a period of proliferation, which generates a pool of precursor cells. Selected cells from this pool exit the cell cycle and initiate a complex program of gene expression that will result in the formation of the mature neuron.

The Drosophila retina is a highly tractable model for studying the relationship between growth and neuronal differentiation (Wolff and Ready 1993). Photoreceptor (PR) differentiation in Drosophila is initiated at the beginning of the third larval instar when a physical indentation, known as the morphogenetic furrow (MF), develops at the posterior of the eye imaginal disc. Over a period of ~48 hr the MF sweeps anteriorly leading to the formation of PR preclusters. The MF is initiated by the morphogen Hedgehog (Hh) and is propagated anteriorly through a combination of Hh and Decapentapalegic (Dpp) signaling (Voas and Rebay 2004). Posterior to the MF, PRs are specified sequentially through reiterative use of the Notch and EGFR pathways (Brennan and Moses 2000; Voas and Rebay 2004).

As in other neurogenic contexts, neuronal differentiation in the Drosophila eye is a temporally restricted process. Patterning of the mature cluster of eight PRs is highly stereotyped with each row forming about every 2 hr (Figure 1A) (Wolff and Ready 1993). The mechanism underlying the temporal control of PR differentiation has proven elusive. Several models have been proposed including control by receptor-mediated cell–cell interactions and intrinsic or extrinsic cellular clocks (Freeman 1997; Brennan and Moses 2000; Voas and Rebay 2004). We found that the conserved InR/TOR pathway plays a key role in controlling the timing of neuronal differentiation in Drosophila (Bateman and McNeill 2004). Using mutants in various components of the InR/TOR pathway, we showed that activation of this pathway causes precocious differentiation of neurons. Conversely, inhibition of InR/TOR signaling significantly delays neurogenesis. How the InR/TOR pathway regulates neuronal differentiation is unclear.

Temporal control of neuronal differentiation is a property of the entire InR/TOR pathway. Ligand binding to the InR causes recruitment and phosphorylation of the insulin receptor substrate (IRS) and subsequent activation of PI3K, which catalyzes the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the membrane (Leevers and Hafen 2004).

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PDK1 and PKB/AKT, both PH domain-containing kinases, become membrane localized by their interaction with PIP3 where PKB/AKT can be fully activated. InR signaling controls growth and proliferation through the inhibition of the GTPase activating protein (GAP) TSC2 (Gao and Pan 2001; Potter *et al.* 2001; Tapon *et al.* 2001; Cai *et al.* 2006). TSC2 inhibits the activity of the small GTPase Rheb, which activates TOR (Long *et al.* 2005). TOR is a phosphatidylinositol kinase-related kinase that is part of a complex (TORC1) that controls growth through the regulation of ribosome biogenesis and translation via S6K and eIF4E, respectively (Inoki and Guan 2006; Wullschleger *et al.* 2006). TOR is also a component of the TORC2 complex. TORC2 is insensitive to rapamycin and has recently been shown to phosphorylate AKT at Ser473 (Sarbassov *et al.* 2005; Guertin *et al.* 2006b). TOR has other functions including the regulation of microautophagy and fat metabolism (Rusten *et al.* 2004; Scott *et al.* 2004). In addition, inhibition of TOR by treatment with rapamycin elicits a transcriptional response involving several hundred genes (Peng *et al.* 2002; Guertin *et al.* 2006a). Recently a negative feedback loop in which S6K regulates IRS, both transcriptionally and by phosphorylation, has been shown to exist in both Drosophila (Radimerski *et al.* 2002) and mammalian systems (Harrington *et al.* 2004; Shah *et al.* 2004; Um *et al.* 2004).

What is the mechanism by which InR signaling controls the timing of neuronal differentiation? In mammalian systems activation of insulin/IGF receptor tyrosine kinases causes activation of both PI3K and Ras/mitogen-activated protein kinase (MAPK) pathways (Baltensperger *et al.* 1993; Skolnik *et al.* 1993; Downward 2003). Ligand binding to the InR results in tyrosine phosphorylation of IRS proteins and/or Shc which, through the adaptor protein Grb2, results in recruitment to the membrane of SOS for the activation of Ras (Baltensperger *et al.* 1993; Skolnik *et al.* 1993). However, flies expressing a version of the Drosophila IRS *chico,* in which the putative Drk (the Drosophila ortholog of Grb2) binding site had been mutated, are able to fully rescue the growth defects of *chico* flies (Oldham *et al.* 2002). Therefore it is currently unclear whether the InR activates MAPK signaling in Drosophila (Bateman and McNeill 2006).

In the current study we find that differentiation is temporally regulated by TOR and S6K, but not by 4EBP or eIF4E, thus providing the first branch in the differentiation pathway downstream of InR signaling in the eye. We also show that activation of the InR/TOR pathway regulates the expression, at the transcriptional level, of the EGFR pathway components Argos, rhomboid (rho), and pointedP2 (pntP2). Moreover, reducing the level of EGFR signaling, by using a *pntP2* hypomorphic allele, causes a cell-type-specific delay in differentiation, which is identical to that in mutants that inhibit the InR/TOR pathway. Finally we show that the EGFR and InR/TOR pathways genetically interact in controlling the timing of PR differentiation.

MATERIALS AND METHODS

To generate loss-of-function clones, 48- to 72-hr-old larvae were heat-shocked for 1–2 hr at 37°. Overexpression clones were generated using the "flp-out" technique (Neufeld *et al.* 1998), where 48- to 60-hr-old larvae were heat-shocked for 2.5 hr at 37°. Third instar eye discs were fixed in PBSA/4% formaldehyde (EMS Scientific) for 45 min, washed in PBSA/0.1% TritonX100 (Sigma, St. Louis) and incubated overnight with primary antibody. Primary antibodies were used as follows: mouse and rabbit anti-GFP (Molecular Probes, Eugene, OR; 1:1000), rabbit anti-Bar (a gift from K. Saigo; 1:200), mouse anti-Prospero (DHSB; 1:10), guinea pig anti-Senseless (a gift from H. Bellen; 1:1000), mouse anti-β-galactosidase (Roche, Indianapolis; 1:1000), rabbit anti-Spalt (a gift from R. Barrio; 1:500), mouse anti-Rough (DSHB; 1:100), mouse anti-Cut (DSHB; 1:20), and mouse anti-Argos (DSHB; 1:100). Secondary antibodies were from Jackson Laboratories (West Grove, PA). After staining, discs were mounted in Vectastain (Vector Laboratories, Burlingame, CA) and analyzed with a Zeiss confocal microscope or a Zeiss Apotome.

To quantify *eIF4E* mutant growth rates the mutant clone area relative to the twin-spot area was quantified using ImageJ and in three independent clones for each genotype.

The following stocks were kindly provided to us: The *pten* flies were from Sally Leevers and *tsc1* flies from Nic Tapon. The *s6k, tsc2* stock was from D. J. Pan. The *Rheb* stocks were from Ernst Hafen. The UAS-4EBP stock was from Nahum Sonenberg. *pnt* stocks were from Christian Klämbt. The rho^{X81} stock was from Matthew Freeman. *eIF4E* (11720), aos^{W11} (2513), and *TOR* (7014) mutants were from The Bloomington Stock Center. Genotypes for generating clones were as follows:

tsc1, *Rheb* mutant clones: y, *w, hs-flp*; FRT82, $dRheb^{2D1}$, $tsc1^{2G3}$/FRT82B, Ubi-GFP.
tsc2 mutant clones: y, *w, hs-flp; gig*^{56}, FRT80/FRT80, Ubi-GFP.
tsc2 mutant clones with pntP2-LacZ: y, *w, hs-flp; gig*^{56}, FRT80, pnt^{1277}/FRT80, Ubi-GFP.
s6k mutant clones: *y, w, hs-flp; s6k*^{l1}, FRT80B/FRT80, P{LacW}RpL14, eGFP.
s6k, tsc2 mutant clones: y, *w, hs-flp; gig*^{192}, *s6k*^{l1}, FRT80/FRT80, Ubi-GFP.
eIF4E mutant clones: y, *w, hs-flp; eIF4E* ^{07238}, FRT80/FRT80, arm-LacZ or y, *w, hs-flp; eIF4E*$^{715/13}$, FRT80/FRT80, arm-LacZ.
eIF4E, tsc2 mutant clones: y, *w, hs-flp; eIF4E* ^{07238}, *gig*^{56}, FRT80/FRT80, P{LacW}RpL14, eGFP.
4EBP overexpression clones: y, *w, hs-flp;* UAS-4EBP/act>y>Gal4, UAS-GFP.
tsc1 mutant clones: y, *w, hs-flp; tsc1*^{Q87X}, FRT82B/FRT82B, Ubi-GFP.
Rheb mutant clones: *hs-flp;* $Rheb^{2D1}$, FRT82/82FRT, Ubi-GFP, M[95A], Rps63.
pten mutant clones: y, *w, hs-flp;* $pten^{1}$, FRT40/FRT40, Ubi-GFP.
pten mutant clones with aos-LacZ: y, *w, hs-flp;* $pten^{1}$, FRT40/FRT40, Ubi-GFP; aos^{W11}/+.
Rheb mutant clones with aos-LacZ: *hs-flp;* aos^{W11}, $Rheb^{2D1}$, FRT82/82FRT, Ubi-GFP, M[95A], Rps63.
pten mutant clones with rho-LacZ: y, *w, hs-flp;* $pten^{1}$, FRT40/FRT40, Ubi-GFP; rho^{X81}/+.
pten mutant clones with pntP2-LacZ: y, *w, hs-flp;* $pten^{1}$, FRT40/FRT40, Ubi-GFP; pnt^{1277}/+.
TOR mutant clones: *y, w, hs-flp;* $TOR^{\Delta P}$, FRT40A/FRT40, Ubi-GFP; pnt^{1277}/+.

pntP2 hypomorph clones: *y, w, hs-flp;* FRT82, pnt^{1230}/FRT8, Ubi-GFP.
Rheb, pntP2 mutant clones: *hs-flp;* pnt^{1230}, $Rheb^{2D1}$, FRT82/82FRT, Ubi-GFP, M[95A], Rps63.
UAS-Dp110, *pntP2* clones: hs-flp, UAS-GFP; UAS-Dp110; tub-Gal80, FRT82, pnt^{1230}/FRT82, tub-Gal80.
UAS-pntP2 clones: hs-flp; act>y>Gal4, UASGFP; UAS-pntP2.
$EGFR^{ACT}$ clones: hs-flp; act>y>Gal4, UASGFP; UAS-$EGFR^{ACT}$.

RESULTS

The InR controls differentiation through a pathway including TOR and S6K, but not 4EBP/eIF4E: We have shown previously that *tsc1* loss-of-function (LOF) clones cause precocious differentiation of PRs in the developing eye (Bateman and McNeill 2004). TSC1, together with TSC2, functions as a GAP for the small GTPase Rheb. We found that loss of *Rheb* causes a strong delay in differentiation suggesting that TSC1/2 acts upstream of Rheb in controlling differentiation as it does in growth (Saucedo *et al.* 2003; Zhang *et al.* 2003). However, TSC1 has targets other than Rheb and can activate RhoGTPase and inhibit Rac1 through interaction with the ERM family of actin binding proteins (Lamb *et al.* 2000; Astrinidis *et al.* 2002; Goncharova *et al.* 2004). Therefore we asked whether TSC1 is able to affect differentiation independently of Rheb. To do this we generated *Rheb, tsc1* double-mutant clones and observed the differentiation phenotype by staining with anti-Prospero (Xu *et al.* 2000). If Rheb is absolutely required for regulation of differentiation by TSC1 then *Rheb, tsc1* double-mutant clones should have a similar phenotype to *Rheb* clones. Alternatively, if the TSC1/2 complex is able to regulate differentiation independent of Rheb, then the delayed differentiation phenotype caused by loss of Rheb should be abrogated in *Rheb, tsc1* clones. *Rheb, tsc1* double-mutant clones show a strong delay in differentiation (Figure 1B), similar to that seen in *Rheb* clones (Figure 2, C and D). This result suggests that the primary target of TSC1/2 in controlling the timing of neuronal differentiation is Rheb.

The TSC1/2 complex and Rheb regulate TOR (Leevers and Hafen 2004). TOR is part of the TORC1 complex, controls growth by phosphorylation of S6K and 4EBP, which in turn affect translation and ribosome biogenesis by regulating RpS6 and eIF4E, respectively (Inoki and Guan 2006; Wullschleger *et al.* 2006). We asked whether S6K and 4EBP are also able to control neuronal differentiation. *s6k* LOF clones do cause a slight delay in differentiation (Figure 1C), which is much weaker than the delay seen in *Rheb* or *TOR* LOF clones (Figure 2, C and D; (Bateman and McNeill 2004). To determine whether S6K mediates the precocious differentiation phenotype seen in *tsc2* clones (Figure 1D) we generated *s6k, tsc2* double-mutant clones. These clones have a wild-type differentiation phenotype (Figure 1E), indicating that S6K acts either downstream or in parallel to TSC2 in controlling differentiation.

TOR also controls growth via the translation initiation factor eIF4E and its inhibitory binding partner 4EBP. Homozygous *eIF4E* Drosophila arrest growth during larval development (Lachance *et al.* 2002). Lachance *et al.* (2002) however did not determine whether *eIF4E* mutant cells have a growth defect. To assess this we made LOF clones of cells using either weak ($eIF4E^{07238}$) or strong ($eIF4E^{715/13}$) *eIF4E* alleles. Clones made using $eIF4E^{07238}$ had a mild but significant growth defect (mean clone size = 67% ± 1% size of twin spot, $n = 3$; supplemental Figure 1), while clones made using $eIF4E^{715/13}$ had a severe growth defect (Figure 1F, compare clone to twin-spot size; mean clone size = 8.7% ± 2% size of twin spot, $n = 3$). Control clones made using a wild-type FRT chromosome were a similar size to the twin spot (mean clone size = 98% ± 1% size of twin spot, $n = 3$) as expected. Surprisingly, neither $eIF4E^{07238}$ (supplemental Figure 1) nor $eIF4E^{715/13}$ LOF clones have any effect on differentiation in posterior (Figure 1F) or anterior clones close to the MF (Figure 1G). Also, $eIF4E^{07238}$, *tsc2* mutant clones have a similarly strong precocious differentiation phenotype to *tsc2* clones (supplemental Figure 1), further suggesting that *eIF4E* is not required for InR/TOR-dependent control of PR differentiation. We also analyzed the differentiation phenotype of the eIF4E inhibitory binding partner 4EBP. In accordance with our results with *eIF4E*, overexpression of *4EBP* also has no effect on differentiation (Figure 1H). In addition, we do not observe any differentiation phenotype in clones of wild-type cells generated in a background heterozygous for a ribosomal subunit dominant mutation (a *Minute* mutant; data not shown), confirming that alteration of the overall translation rate does not affect differentiation. Taken together these data suggest that the control of the timing of neuronal differentiation is regulated by S6K and is independent of 4EBP/eIF4E, while growth is controlled by both these factors.

InR/TOR signaling controls the timing of the differentiation of a subset of photoreceptors: Each ommatidium in the Drosophila eye consists of eight photoreceptor (PR) neurons and 12 accessory cells. We have shown that the InR/TOR pathway controls the timing of differentiation of PRs 1, 6, and 7 and cone cells, but does not affect PR 8 (Bateman and McNeill 2004). The differentiation of PR 8 is followed by the sequential differentiation of PRs 2/5, then PRs 3/4, and finally PRs 1, 6, and 7 (Figure 1A). To determine whether the differentiation of PRs 2–5 is also regulated by the InR/TOR pathway we used antibodies against the transcription factors Rough (Kimmel *et al.* 1990) and Spalt (Barrio *et al.* 1999) to analyze the differentiation of PRs 2/5 and 3/4, respectively. If InR/TOR signaling does regulate the differentiation of PRs 2–5 we would expect activation of the pathway by loss of *tsc1* to cause

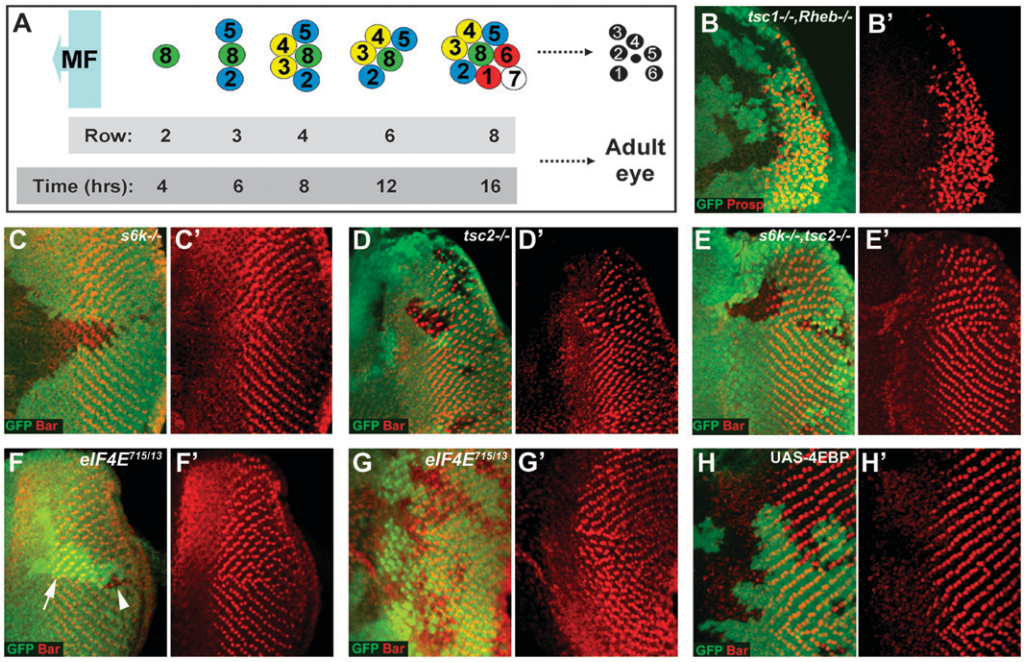

FIGURE 1.—InR and TOR signaling act through S6K, but not eIF4E to control the timing of neuronal differentiation. (A) Schematic showing the spatiotemporal nature of PR differentiation in the Drosophila eye imaginal disc. MF, morphogenetic furrow. (B and B′) $tsc1^{2G3}$, $Rheb^{2D1}$ double-mutant clones have an identical delay in differentiation (stained for Prospero expression, shown in red) to $Rheb^{2D1}$ clones (Figure 2C). (C and C′) Loss of *S6k* causes a slight delay in the differentiation of PR 7 and cone cells (stained for Bar expression, shown in red). (D and D′) *tsc2* (gig^{56}) clones cause precocious differentiation of PRs 1 and 6 (stained for Bar expression, shown in red). (E and E′) The precocious differentiation phenotype of *tsc2* cells is suppressed in *tsc2* (gig^{192}), $s6k^{11}$ clones (Bar staining in red). (F and G) $eIF4E^{715/13}$ LOF clones inhibit growth resulting in small clones, compare clone (arrowhead) to twin spot (arrow) size in F, but do not affect differentiation in posterior clones generated using hs-flp (F and F′) or clones close to the MF, generated using ey-flp (G and G′), (Bar staining in red). (H and H′) overexpression of 4EBP (shown by the presence of GFP staining) does not have any affect on differentiation of PRs 1 and 6 (stained for Bar expression, shown in red). LOF clones in B–G are marked by the loss of GFP (shown in green). Anterior is to the left in all panels.

precocious differentiation of these PRs. Both Rough and Spalt staining appeared normal within *tsc1* clones (Figure 2B and data not shown), suggesting that the InR/TOR pathway does not affect the timing of differentiation of PRs 2/5 or PRs 3/4.

We were concerned that since PRs 2/5 and 3/4 differentiate close to the morphogenetic furrow (rows 3 and 4, respectively, Figure 1A), that it might be difficult to resolve cells which are precociously differentiating. To overcome this issue we made *Rheb* LOF clones to determine whether there is any delay in the differentiation of PRs 2–5 when InR/TOR signaling is inhibited. Differentiation of PRs 1, 6, and 7 and cone cells is strongly delayed in *Rheb* clones (Figure 2, C and D and (BATEMAN and MCNEILL 2004), however, both Rough (PRs 2 and 5) and Spalt (PRs 3 and 4) staining is unaffected in these clones (Figure 2, E and F). Therefore temporal control of differentiation by the InR/TOR pathway in the developing eye is stage/cell type specific: the late differentiating PRs 1, 6, and 7 and cone cells are dependent on the InR/TOR pathway, while the early differentiating PRs 2–5 and 8 are independent of InR/TOR signaling.

Transcription of Argos, a reporter of EGFR signaling activity, is regulated by the InR/TOR pathway: The stage/cell-type-specific nature of the temporal control of differentiation suggests that the InR/TOR pathway achieves this regulation through a novel mechanism. To investigate this we asked whether any of the pathways known to be important for PR differentiation are affected by changes in InR/TOR signaling. Since the passage of the MF is unaffected by the InR/TOR pathway it seemed unlikely that Dpp, Hh, or Wingless signaling were being affected. Next we asked whether EGFR signaling is regulated by the InR/TOR pathway in the developing eye. We had previously analyzed EGFR signaling activity in two ways. First we stained with an antibody against dual phosphorylated MAPK (dpERK), which gives a direct readout of EGFR signaling levels (GABAY *et al.* 1997). Second we analyzed the level of the E26 transformation-specific sequence (ETS) protein Yan, whose accumulation in the nucleus is dependent on its phosporylation state and hence the level of EGFR activity (TOOTLE *et al.* 2003; SONG *et al.* 2005). Neither dpERK nor Yan staining are affected by activation of InR/TOR signaling (BATEMAN and MCNEILL 2004). However, we had not tested whether EGFR signaling is being affected downstream or in parallel to MAPK and Yan.

To test whether there is any overall activation of EGFR signaling by the InR/TOR pathway we looked at the expression of Argos. Argos is a secreted protein that functions as an inhibitory ligand of the EGFR (FREEMAN *et al.* 1992b). *argos* expression is induced by EGFR activation in differentiating cells and is thought to result in a feedback loop that inhibits the differentiation of surrounding cells (GOLEMBO *et al.* 1996). As a

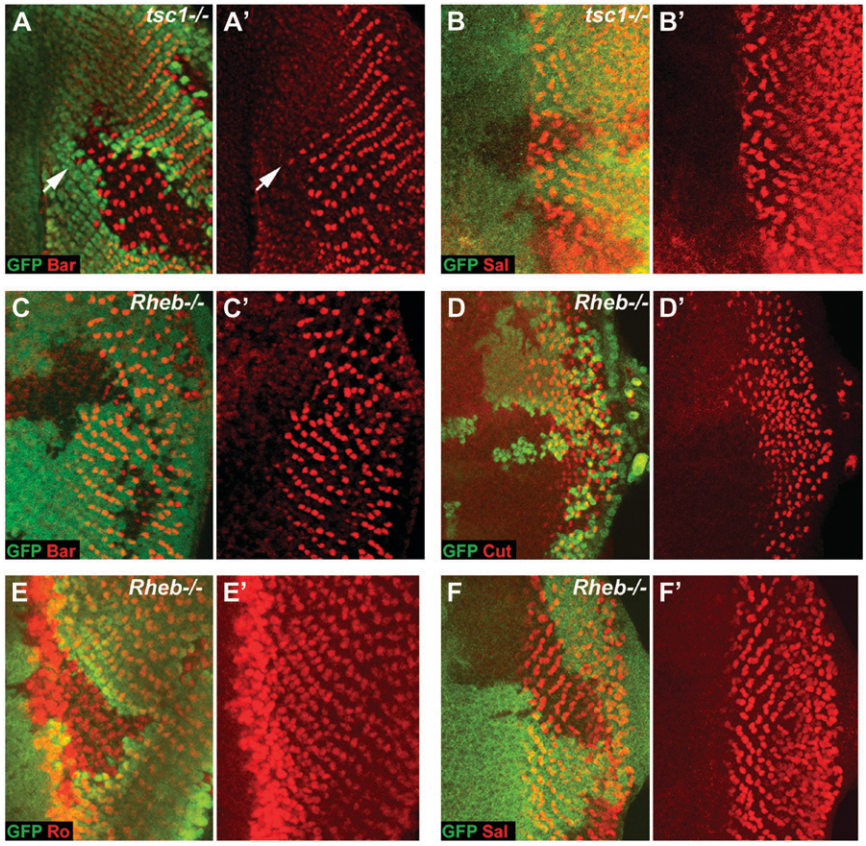

FIGURE 2.—InR/TOR signaling controls the differentiation of specific cell types in the developing eye. (A and A′) Cells mutant for *tsc1* (*tsc1*^{Q87X}) show precocious differentiation of PRs 1 and 6 (stained for Bar expression, shown in red) ahead of the wild-type differentiation front; arrow indicates an example of a precociously differentiated PR. (B and B′) Differentiation of PRs 3/4 (stained for Spalt expression, shown in red) is unaffected in *tsc1*^{Q87X} clones. (C and D) Differentiation of PRs 1 and 6 (stained for Bar expression, shown in red in C and C′) and cone cells (stained with Cut, shown in red in D and D′) is strongly delayed in *Rheb*^{2D1} clones. (E and F) PR 2/5 (stained for Rough expression, shown in red in E and E′) and PR 3/4 (stained for Spalt expression, shown in red in F and F′) differentiation is unaffected in *Rheb*^{2D1} clones. LOF clones in all panels are marked by the loss of GFP staining (shown in green). Anterior is to the left in all panels.

consequence of its dependence on EGFR activation Argos is strongly expressed in developing PRs as they differentiate (FREEMAN *et al.* 1992b). To analyze the expression of Argos in cells in which InR/TOR signaling is activated we stained *pten* LOF clones with an Argos monoclonal antibody. Although Argos stains poorly in imaginal discs we see a consistent increase in Argos accumulation in *pten* clones (Figure 3A).

Next we asked whether the ability of the InR/TOR pathway to modulate Argos levels is caused by changes in *argos* gene expression. This result would indicate that EGFR signaling is being affected, rather than a stabilization of Argos post-transcriptionally. To address this we used the *argos*^{W11} lacZ reporter line (FREEMAN *et al.* 1992a,b). Using *argos*^{W11} we observed a strong increase in *argos* expression in *pten* LOF clones (Figure 3B). Interestingly, in *pten* clones that cross the MF, strong precocious expression of *argos* is seen in the mutant cells (Figure 3B). To determine whether inhibition of the InR/TOR pathway can regulate *argos* expression we generated *Rheb* clones in larvae carrying the *argos*^{W11} allele. Loss of *Rheb* causes a strong decrease in *argos* expression in differentiating cells (Figure 3C). Thus both positive and negative regulators of the InR/TOR signaling pathway lead to alterations in *argos* expression.

Since Argos is also an inhibitory ligand of the EGFR (FREEMAN *et al.* 1992b), we analyzed the expression of *rhomboid-1* (*rho*) as an independent readout of EGFR activity. *rho* expression was monitored using the X81 enhancer trap line which is expressed strongly in PRs 2/5 and 8 (FREEMAN *et al.* 1992a). In accordance with the *argos* data, *rho* expression is upregulated in *pten* LOF clones (Figure 3D). These changes appear to be specific since the expression of several other cell fate genes is unaffected by changes in InR/TOR signaling (BATEMAN and MCNEILL 2004), including the Notch ligand Delta (supplemental Figure 2). In conclusion, these data suggest that there is crosstalk between InR/TOR signaling and the EGFR pathway and that this occurs downstream of MAPK.

Expression of pntP2 is regulated by InR/TOR signaling: *argos* expression is activated by the ETS transcription factor *pointed* (*pnt*). *pnt* is expressed as two alternatively spliced isoforms, P1 and P2, which share a C-terminal region that contains the ETS motif (SCHOLZ *et al.* 1993). *pntP2* is expressed specifically in the embryonic midline glial cells (KLAMBT 1993), and *argos* expression is lost in these cells in *pointed* (*pnt*) mutant embryos (SCHOLZ *et al.* 1997). Activation of the EGFR results in phosphorylation of MAPK, which enters the nucleus and phosphorylates pntP2 (BRUNNER *et al.* 1994; O'NEILL *et al.* 1994). In the eye imaginal disc *pntP2* is expressed in precursor cells posterior to the MF and in PRs 1, 6, and 7 and cone cells (BRUNNER *et al.* 1994). Since *argos* is a transcriptional target of pntP2 we wondered whether pntP2 expression might also be regulated by InR/TOR signaling. To test whether *pntP2* expression is regulated by InR/TOR signaling we used

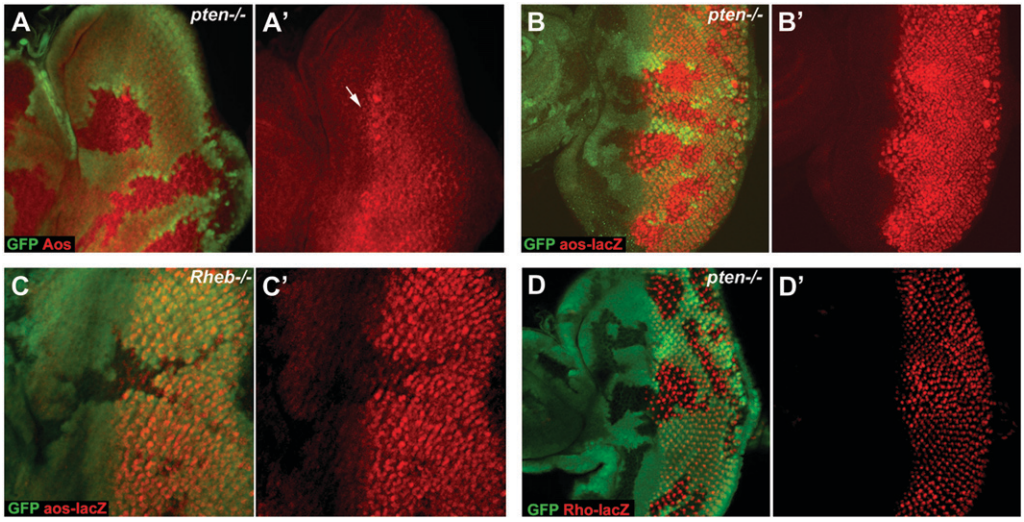

Figure 3.—*argos* and *rho* expression is regulated by InR/TOR signaling in developing neurons. (A and A′) The level of Argos protein (detected using an anti-Argos monoclonal antibody, shown in red) is increased in *pten*^{1} mutant cells (marked by loss of GFP staining). Note how Argos staining is seen ahead of the normal expression front (marked with an arrow). (B and C) *argos* expression is regulated by InR/TOR signaling at the level of transcription. Expression of β-galactosidase (stained with an anti-β-galactosidase antibody, shown in red) from the *P*{lwB}-element insertion in *argos* (*aos*^{w11}) is upregulated in *pten*^{1} clones (B and B′) and downregulated in *Rheb*^{2D1} clones (C and C′). (D and D′) *rho* expression (using the *rho*^{X81} reporter, detected by staining with a anti-β-galactosidase antibody, shown in red) is upregulated in *pten*^{1} clones. Clones are marked by loss of GFP staining and anterior is to the left in all panels.

the *pnt*^{1277} allele which contains a *P*{LacW} element within the first, noncoding exon of *pntP2* (Scholz *et al.* 1993). Using *pnt*^{1277} we observe a strong increase in *pntP2* expression in *pten* LOF clones (Figure 4A). Interestingly, the increase in *pntP2* expression differs spatiotemporally across the field of differentiating cells. *pntP2* expression is increased most strongly in cells as they differentiate, but this increase is lost once the cells become more mature. Moreover, dramatic precocious expression of *pntP2* is observed in *pten* clones that span the MF (Figure 4A). Importantly, *pntP2* expression is also upregulated in undifferentiated cells around the MF, suggesting that the increase in expression is not simply an indirect consequence of the precocious differentiation of PRs. We also observe a similar upregulation of *pntP2* expression in clones that have activated InR/TOR signaling due to loss of *tsc2* (Figure 4B). The increase in *pntP2* expression is not a result of a general increase in transcription due to increased growth, since we do not see increased expression of several other markers of PR cell fate (Bateman and McNeill 2004). To examine the effect of blocking InR/TOR signaling we examined *pntP2* expression in cells mutant for *TOR*. LOF clones of *TOR* show decreased expression of *pntP2* (Figure 4C). Therefore *pntP2* expression is sensitive to both activation and inhibition of InR/TOR signaling. To determine whether this property is specific to the eye we looked at *pntP2* expression in *pten* clones in the leg and eye discs. We did not observe any change in *pntP2* expression in these clones (supplemental Figure 3), suggesting either that InR/TOR regulation of *pntP2* is specific to the developing eye (perhaps requiring specific factors expressed close to the MF) or that the spatiotemporal nature of eye development in Drosophila makes it possible to observe changes that cannot be resolved in other imaginal discs.

Reducing EGFR signaling phenocopies loss of Rheb or TOR in developing PRs: *Argos*, *rho*, and *pntP2* expression levels are all regulated by InR/TOR signaling, suggesting crosstalk between InR/TOR and EGFR pathways. However, complete loss of EGFR or pntP2 activity (using null alleles) completely *blocks* the differentiation of all PRs except PR 8 (data not shown; (Baonza *et al.* 2001, 2002; Yang and Baker 2003), whereas inhibition of the InR/TOR pathway causes a *delay* only in the differentiation of PRs 1, 6, and 7 and cone cells (Figure 2). To reconcile these observations we wondered whether a reduction, rather than a complete loss in EGFR activity would cause the same cell-type-specific delay in differentiation as inhibition of the InR/TOR pathway.

To determine the affect of reducing EGFR signaling levels we used a hypomorphic allele of *pntP2* (*pntP2*^{1230}), which was generated by the imprecise excision of a *P* element in the first, noncoding exon of *pntP2* (Klambt 1993). We stained *pntP2*^{1230} clones with the same panel of markers that we had used to analyze the differentiation phenotype of InR/TOR pathway mutants (Figure 2). Interestingly the PR differentiation phenotype in *pntP2*^{1230} clones is identical to that in *Rheb* or *TOR* LOF clones (compare Figure 5 to Figure 2). Specifically, PR 8 (stained for Senseless expression; Figure 5A), PRs 2/5 (stained for Rough expression; Figure 5B), and PRs 3/4 (stained for Spalt expression; Figure 5C) differentiate normally in *pntP2*^{1230} clones. In contrast the differentiation of PRs 1 and 6 (stained for Bar expression; Figure 5D), PR 7 (stained for Prospero expression; Figure 5E), and cone cells (stained for Prospero expression; Figure 5E and Cut expression; Figure 5F) are strongly

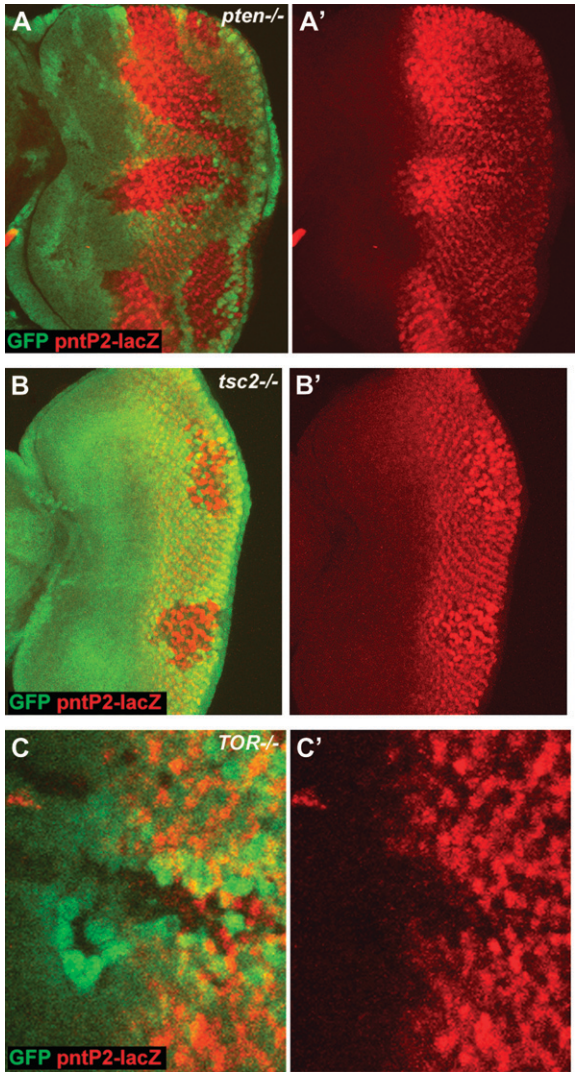

FIGURE 4.—pntP2 expression is regulated by InR/TOR signaling. (A and B) *pntP2* transcription, detected by staining for β-galactosidase in flies carrying a *P*{LacW} element in *pntP2* (*pnt*^{1277}), is upregulated and precocious in *pten*^{1} (A and A′) and *tsc2* (*gig*^{56}) clones (B and B′). Note that the disc shown in B is a younger disc and so *pntP2* is upregulated more posteriorly. Conversely *pntP2* transcription is downregulated in *TOR*$^{\Delta D}$ clones (C and C′). LOF clones in A–C are marked by the loss of GFP staining (shown in green). Anterior is to the left in all panels.

delayed but not completely blocked. The phenotypic similarity between PRs with reduced EGFR signaling and PRs in which InR/TOR signaling is inhibited is consistent with InR/TOR signaling modulating EGFR transcriptional outputs to control neuronal differentiation.

InR/TOR and EGFR signaling interact genetically: Since reducing EGFR pathway activity through pntP2 phenocopied inhibition of the InR/TOR pathway we wondered whether these two pathways could interact genetically. To test this we generated clones that were double mutant for *pntP2*^{1230} and *Rheb*^{2D1}. Inhibition of differentiation in these clones (Figure 6B) was much more severe than in *pntP2* (or *Rheb*) single mutant clones (Figure 6A). *pntP2*^{1230}, *Rheb*^{2D1} double-mutant clones block rather than delay the differentiation of PRs 1 and 6 (Figure 6B). Conversely, when we overexpressed Dp110 in *pnt*^{1230} clones using the mosaic analysis with a repressible cell marker (MARCM) technique (LEE and LUO 1999), the delay in the differentiation of PRs 1 and 6 was much less severe (Figure 6D) than in *pnt*^{1230} clones alone (Figure 6C) and the precocious differentiation normally seen with Dp110 overexpression was completely suppressed, strongly suggesting that *pntP2* acts downstream of Dp110. These data demonstrate that the InR/TOR and EGFR pathways can interact genetically and are consistent with the regulation of neuronal differentiation by the InR/TOR through modulation of EGFR transcriptional output.

DISCUSSION

Tight coordination of growth and differentiation is essential for normal development. We have previously shown that InR/TOR signaling controls the timing of neuronal differentiation (BATEMAN and MCNEILL 2004) in the eye and leg in Drosophila. Here we demonstrate that the InR/TOR pathway regulates neuronal differentiation in an S6K-dependent, but 4EBP/eIF4E-independent manner. Previously we were unable to determine whether InR/TOR signaling was acting downstream or in parallel to the EGFR/MAPK pathway. Using *argos* and *rho* as reporters we have shown that the InR/TOR pathway is able to regulate EGFR/MAPK signaling downstream of MAPK. Moreover, *pntP2* expression is up- and downregulated by activation or inhibition of InR/TOR signaling, respectively, and InR/TOR and EGFR pathways interact through *pntP2*. Taken together our data suggest that temporal control of differentiation by the InR/TOR pathway is achieved by modulation of EGFR pathway transcriptional targets in differentiating PRs.

TOR is part of two multimeric complexes (TORC1 and TORC2) and is a core component of the InR pathway (INOKI and GUAN 2006; WULLSCHLEGER *et al.* 2006). TORC1 activity is regulated by nutrient and energy levels (HARA *et al.* 1998; INOKI *et al.* 2003) providing a conduit for hormonal and catabolic cellular inputs. Growth is regulated by two downstream targets of TORC1: S6K and 4EBP. Our data demonstrate that upstream of TORC1, differentiation and growth are regulated by the same factors. Downstream of TORC1, differentiation and growth differ significantly in that loss of *s6k*, but not *eIF4E* (or overexpression of 4EBP) affects differentiation. eIF4E regulates 7-methyl-guanosine cap-dependent translation and is the rate-limiting factor in translation initiation (RICHTER and SONENBERG 2005). Our finding that eIF4E does not affect differentiation suggests that the temporal control of differentiation is not based on a translation initiation-dependent mechanism. Strikingly, we show that loss of *s6k* blocks

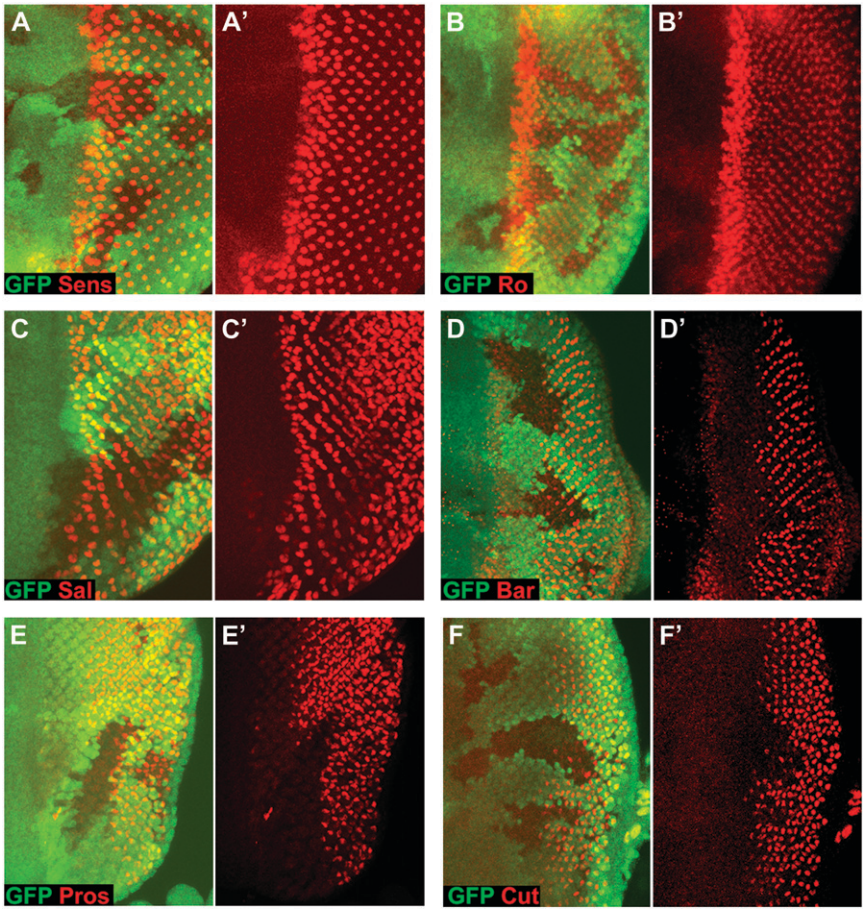

Figure 5.—Reducing EGFR signaling phenocopies the differentiation phenotype of loss of Rheb or TOR. pntP2 hypomorphic clones made using the allele $pntP2^{1230}$ show cell-type-specific delays in PR differentiation identical to those seen in LOF clones of positive effectors of InR/TOR signaling such as *Rheb* and *TOR*. (A–C) $pntP2^{1230}$ clones have no effect on the differentiation of PR 8 (stained for Senseless expression, shown in red in A and A′), PRs 2/5 (stained for Rough expression, shown in red in B and B′) or PRs 3/4 (stained for Spalt expression, shown in red in C and C′). Note that Spalt staining shows a delay toward the posterior of the disc where the antibody also stains PRs 1 and 6. In contrast $pntP2^{1230}$ clones show a strong delay in the differentiation of PRs 1 and 6 (stained for Bar expression, shown in red in D and D′), PR 7 (stained for Prospero expression, which is also expressed in cone cells, shown in red in E and E′) and cone cells (stained for Cut expression, shown in red in F and F′ and Prospero expression, shown in red in E and E′). LOF clones in all panels are marked by the loss of GFP staining (shown in green). Anterior is to the left in all panels.

the precocious differentiation induced by loss of *tsc2*. Given the relatively weak effects of loss of *s6k* this may seem surprising. However, the degree of suppression is similar to the effect of loss of *s6k* on the overgrowth phenotype caused by loss of *tsc2*, namely, *tsc2, s6k* double-mutant cells are the same size as wild-type cells (Gao *et al.* 2002). Although loss of *eIF4E* has no affect on differentiation it may act redundantly with another factor, such as *s6k*. Testing this hypothesis though is technically challenging since the Drosophila genome contains eight different *eIF4E* isoforms (Hernandez *et al.* 2005). It will be interesting in future to test whether any of these isoforms regulate differentiation or alternatively whether *eIF4E* and *s6k* act redundantly. Although further work is required to determine the precise relationship between S6K and the InR/TOR

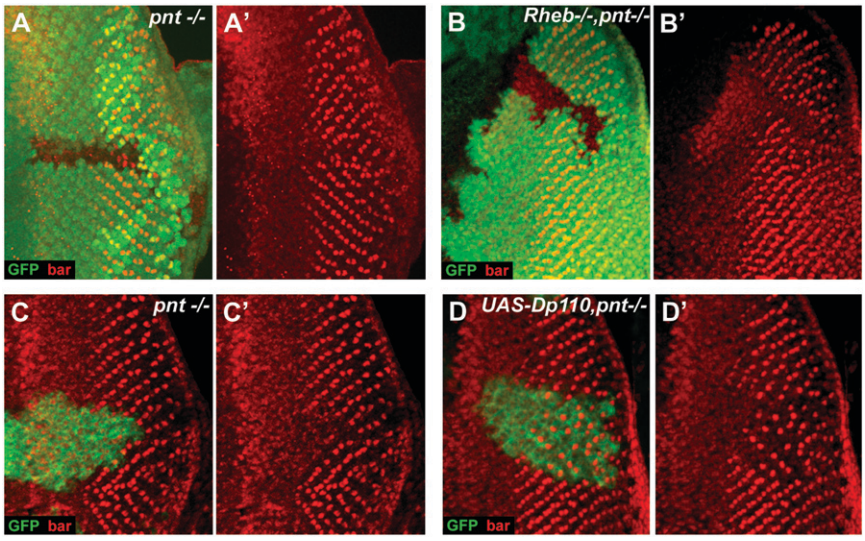

Figure 6.—InR/TOR and EGFR signaling interact genetically. (A and B) Differentiation of PRs 1 and 6 is delayed in pnt^{1230} clones (A and A′) and blocked in pnt^{1230}, $Rheb^{2D1}$ clones (B and B′). (C and D) Using the MARCM system differentiation of PRs 1 and 6 is delayed in pnt^{1230} clones (C and C′), whereas the delay is significantly weaker in pnt^{1230} clones overexpressing Dp110 (D and D′). Mutant cells are marked by the absence of GFP in A and B, but by the presence of GFP in C and D. PRs 1 and 6 are shown by Bar staining (red) in all panels.

pathway, our data point to a critical role of S6K in coordinating neuronal differentiation and growth.

As in other neuronal systems, differentiation of PRs in the Drosophila eye occurs in a stereotyped manner. The advantage of the Drosophila retina as an experimental system is that the PRs differentiate spatiotemporally. Using this feature, as well as a series of cell-type-specific antibodies, we have demonstrated that InR/TOR signaling is selective in the cell-types that it affects. The differentiation of PRs 2/5, 3/4, and 8 are unaffected by perturbations in InR/TOR signaling, whereas PRs 1, 6, and 7 and cone cells are dependent on this pathway for temporal control of differentiation. Interestingly the affected cells all differentiate after the second mitotic wave. However, we have shown that regulators of the cell cycle do not affect the temporal control of differentiation (Bateman and McNeill 2004). Why then are PRs 1, 6, and 7 and cone cells specifically affected? In cells with increased InR/TOR signaling, the expression of *argos*, *rho*, and *pntP2* is precocious and increased throughout the clone, suggesting that the upregulation of EGFR signaling occurs in all cells. However, decreasing EGFR activity using a hypomorphic *pntP2* allele specifically affects the differentiation of PRs 1, 6, and 7 and cone cells. Interestingly, *pntP2* expression in differentiated cells is also restricted to PRs 1, 6, and 7 and cone cells. These observations suggest that differentiation of PRs 1, 6, and 7 and cone cells is critically dependent on EGFR levels signaling through *pntP2*. Therefore, although activation of InR/TOR signaling causes upregulation of EGFR transcriptional targets in all cells as they differentiate, the phenotypic effect is only seen in PRs 1, 6, and 7 and cone cells since these cells are highly sensitive to EGFR activity signaling through *pntP2*. This possibility is supported by the fact that precocious differentiation caused by overexpression of Dp110 can be suppressed by the simultaneous reduction of *pntP2* levels (Figure 6). The complete suppression of the Dp110 differentiation phenotype by simultaneous reduction of *pntP2* strongly suggests that *pntP2* acts downstream of Dp110 and InR/TOR signaling in a pathway that regulates the temporal control of differentiation. It has been suggested that later differentiating PRs require higher levels of EGFR activity than their earlier differentiating neighbors. In particular, the activation of PR 7 requires both EGFR and Sevenless RTKs (Freeman 1996). In the case of InR/TOR pathway activation it may be that, through its regulation of EGFR downstream targets, the "second burst" of RTK activity is enhanced causing PRs 1, 6, and 7 and cone cells to differentiate precociously. There may also be other as yet unidentified factors through which the InR/TOR pathway controls the expression of Aos and rho in PRs 2–5 and 8.

Activation of insulin and insulin-like growth factor receptors in mammalian systems is well known to elicit a response via the Ras/MAPK pathway (Baltensperger *et al.* 1993; Skolnik *et al.* 1993; Downward 2003;). However, loss of the *InR* in the Drosophila eye does not result in a loss of PRs, a hallmark of the Ras pathway (Brogiolo *et al.* 2001), nor does mutation of the putative Drk binding site in *chico* affect the function of the Drosophila IRS (Oldham *et al.* 2002). In accordance with these data we do not observe any change in dpERK staining when the InR/TOR pathway is activated in the eye disc. Rather than a direct activation of Ras signaling by the InR, our data suggest that in the developing eye crosstalk between these pathways occurs at the level of regulation of the expression of EGFR transcriptional outputs. The most proximal component of the EGFR pathway that is regulated by InR/TOR signaling is *pntP2*. However, our data suggest that temporal control of PR differentiation requires concerted regulation of EGFR transcriptional outputs, since overexpression of *pntP2* alone is not sufficient to cause precocious differentiation, whereas overexpression of activated EGFR is sufficient (supplemental Figure 4). Interestingly, microarray analyses of Drosophila and human cells have shown that the InR/TOR pathway regulates the expression of hundreds of genes (Peng *et al.* 2002; Guertin *et al.* 2006a). The mechanism by which this transcriptional control is exerted has yet to be elucidated. It will be interesting in future to determine the extent of transcriptional crosstalk between InR/TOR and EGFR pathways in developing neurons.

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Cellular and Molecular Life Sciences

Visions & Reflections

Insulin/IGF signalling in neurogenesis

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Introduction

A fundamental challenge during the development of any complex organism is the coordination of proliferation and differentiation. In the case of neurogenesis, cells must exit the cell cycle and undergo a complex programme of gene expression and morphological changes. This requires the action of multiple secreted ligands which, by binding to their target receptors on the cell surface, control the course of neuronal cell fate in a spatiotemporal manner. Neurogenic organs are wholly dependent on prior proliferation to provide enough cells to generate the mature tissue. There are often assumed to be two sets of independent signalling pathways: one which controls proliferation and a second which controls differentiation. In this context, neuronal differentiation might be seen as a default pathway that occurs as a result of growth factor removal. Surprisingly, however, the same pathway often regulates both proliferation *and* differentiation. In this review we discuss the role of the insulin receptor (IR) and the type I insulin-like growth factor receptor (IGF-IR) receptor tyrosine kinases (RTKs) in neuronal differentiation by comparing knowledge about vertebrates with insight gained from studies in *Drosophila*. Evidence from vertebrates and flies suggests that, in certain developmental contexts and cell types, IR/IGF-IR signalling plays an important role in the differentiation of neurons.

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Insulin/IGF signalling in vertebrate neurogenesis

Although the role of IR and IGF-IR signalling in cell proliferation has been clearly demonstrated, the potential role of this group of RTKs in neuronal differentiation has received less attention. Insulin is best known for its role in glucose uptake and metabolism, whereas the insulin-like growth factors (IGFs) are well characterised as growth-promoting peptides [1]. Expression studies of the IR and IGF-IR have demonstrated that both of these RTKs are expressed in the nervous system [2, 3], suggesting that they function in neuronal development. The IR is widely expressed throughout the adult brain and concentrated expression is found in the hypothalamus, olfactory bulb and pituitary [3–5]. In addition, the IGF-IR is expressed in many embryonic tissues but high levels of expression are seen in the developing cerebellum, midbrain, olfactory bulb and the ventral floorplate of the hindbrain [2].
In cultured cells, insulin and IGF-I do not always act as mitogens. For example, in mouse fibroblast cell lines, insulin and IGF-I are very poor mitogens [6]. Insulin and IGF-I can also activate neurogenesis in ex vivo and cultured cell lines [6–11]. H19-7 rat hippocampal cells proliferate at 34 °C in response to serum and differentiate to a neuronal phenotype at 39 °C when treated with basic fibroblast growth factor (bFGF). However, expression of the IGF-IR allows HC19-7 cells to differentiate at 39 °C in response to IGF-I independent of bFGF [9]. In E14 mouse striatal primary neural stem cells (NSCs), the action of insulin/IGF-I to activate either proliferation

or differentiation is dependent on the passage number of the cells. NSCs isolated from neurospheres after two rounds of culture for 1 week differentiate to a neuronal phenotype in response to treatment with IGF-I [7]. Interestingly, the neurogenic action of IGF-I could be potentiated by the addition of brain-derived neurotrophic factor (BDNF), suggesting that these factors can act synergistically to promote differentiation. Conversely, treatment of similar NSCs from primary cultures with IGF-I caused individual cells to proliferate rapidly rather than differentiate [8]. Therefore, the ability of insulin/IGF to promote either differentiation or proliferation depends on the cell type and conditions.

What do the phenotypes of *Ir* and *Igf1r* mutant animals tell us about the role of these RTKs in neurogenesis? *Ir–/–* null mice develop normally but die shortly after birth due to severe diabetic ketoacidosis [12], suggesting that the IR is not required for neuronal development. Moreover, a neuron-specific disruption of the *Ir* gene in mice did not affect brain development or neuronal survival [13]. In contrast, *Igf1r–/–* mice have reduced brain size and altered brain structures, including a marked increase in the density of neural cells in the spinal cord and brainstem [14]. Furthermore, detailed examination of cochlear development has shown that development of this sensory organ is severely impaired in *Igf1r–/–* mice [15]. A significant decrease in the number of auditory neurons along with aberrant expression of early neural markers suggests that neuronal differentiation in the inner ear is delayed in these mice. Recent studies have also shown that IGF-I is required for differentiation of neuroblasts in the otic vesicle in chick [16]. Moreover, differentiation of neurons derived from mouse olfactory bulb stem cells requires IGF-I [17]. Thus, in mice the IGF-IR seems to be essential for correct central nervous system (CNS) development, while the IR may either be redundant or play a more subtle role.

What are the intracellular signalling cascades by which the IR and IGF-IR RTKs have the potential to control differentiation? In mammalian systems, insulin stimulation has been shown to cause activation of the Ras/mitogen-activated protein kinase (MAPK) pathway [18–20]. Activation of MAPK by the IR is independent of the role of this receptor in glucose homeostasis since inhibition of MAPK activation does not affect the metabolic actions of insulin [21]. Ligand binding to the IR results in tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins and/or Shc, which, through the adaptor protein Grb2, results in recruitment to the membrane of SOS for the activation of Ras (Fig. 1) [22, 23]. MAPK activation is the most well defined route by which IR/IGF-IR signalling might control neurogenesis during development. The first *in vivo* evidence for insulin stimulation of Ras came from the demonstration that insulin-induced *Xenopus* oocyte maturation is blocked by an antibody which inhibits Ras [24]. More recently, knock-out mice studies have shown that MAPK activation by insulin *in vivo* is dependent on IRS-1 [25]. In cultured cells, activation of MAPK is required for nerve growth factor (NGF)/epidermal growth factor (EGF)-dependent differentiation of PC12 cells [26]. Activation of MAPK in PC12 cells causes phosphorylation of target transcription factors and consequent reprogramming of gene expression to a neuronal fate [27]. Activation of MAPK by an IR/IGF-IR receptor-dependent mechanism has the potential to activate a similar neurogenic switch in target cells in the developing nervous system.

The other pathway which is activated by insulin/IGF receptor stimulation is PI3K/TOR signalling (Fig. 1). PI3K/TOR kinase signalling is known to regulate growth through the control of ribosome biogenesis and protein synthesis [28]. PI3K catalyses the conversion of PIP2 to PIP3, a process which is reversed by the lipid phosphatase PTEN. Growth control is mediated through TOR by the activation of S6K and the translation initiation factor eIF4E. The possible role of PTEN in the nervous system has been studied by several groups using conditional knock-out strategies. Although PTEN is not essential for cell fate determination in the CNS overall [29, 30], a dramatic effect was observed in glial cells. Yue et al. [31] used GFAP-cre to generate *pten–/–* cells in the CNS and observed premature differentiation of Bergmann glia in the early postnatal brain. The premature differentiation of *pten–/–* glia resulted in layering defects and subsequent aberrant migration of granule neurons. These data support a role for PTEN acting as a positive regulator of differentiation in certain cell types in the brain.

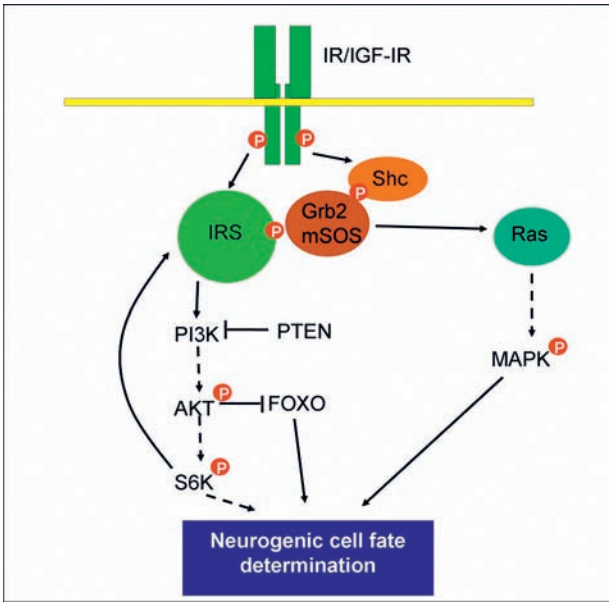

Figure 1. Potential pathways by which insulin/IGF signalling can regulate neurogenesis.

Insulin receptor signalling in *Drosophila*

Unlike vertebrates, *Drosophila* has a single RTK of the insulin receptor family (DInr). Expression of the DInr is ubiquitous during early stages of embryogenesis, but becomes enriched in the developing nervous system [32, 33]. The DInr can be activated by one of seven *Drosophila* insulin-like peptides (DILPS). Three of the DILPS are produced by seven neurosecretory cells within the brain. Flies in which these neurosecretory cells have been ablated are phenotypically similar to *dInr* mutants and have some features that are analogous to diabetes [34]. The DInr is required for growth during development and to attain full adult size [35]. Hypomorphic *dInr* mutants are developmentally delayed and have reduced size due to decreased cell number and cell size [36], suggesting that the role of the DInr during development is analogous to the IGF-IR. *dInr–/–* animals have defects in the development of embryonic central and peripheral nervous systems [32]. Unfortunately, this phenotype has not been studied in detail and so it is not clear whether embryonic neurons in *dInr* mutants are lost due to an inhibition of neurogenesis, proliferation, or indirectly though neuroblast apoptosis. In the developing eye, photoreceptor neurons do not absolutely require the DInr for neurogenesis; however, in the absence of the DInr, neuronal differentiation is significantly delayed [37]. Unlike activation of Ras/MAPK signalling, which is able to induce ectopic neurogenesis in the eye field, activation of DInr signalling modulates the timing of the differentiation programme. These findings suggest that the role of DInr signalling in neuronal differentiation is to act synergistically with other neurogenic pathways, such as EGF receptor (EGFR) signalling.

Does the DInr regulate the same intracellular signal transduction pathways as its mammalian counterparts? In *Drosophila* tissue culture cells, stimulation with mammalian insulin causes rapid phosphorylation of MAPK [38–40]; however, to date this has not been reported *in vivo*. Over-activation of MAPK signalling in the developing eye in *Drosophila* causes the formation of ectopic photoreceptor neurons [41, 42]. Over-expression of the DInr in the eye causes over-proliferation and, although the normal complement of photoreceptors are produced, there is a disruption in the patterning of the eye [36]. Interestingly, the patterning defect caused by over-expression of the DInr is similar to the planar cell polarity defects seen with mutations in EGFR signalling [43, 44], suggesting there may be cross-talk between these two pathways *in vivo*.

Chico, the *Drosophila* IRS, contains conserved putative binding sites for Drk, the homologue of the adaptor protein Grb2 [45]. Oldham et al. [46] generated transgenic flies containing a version of *chico* in which the putative Drk-binding site had been mutated, and found that this mutant was able to fully rescue the growth defects of *chico–/–* flies. In contrast, if the binding site for the regulatory subunit of PI3K (p60) in Chico was mutated, there was a complete loss of function. Why then is the Drk-binding site in the *Drosophila* IRS conserved? It is possible that a low level of MAPK activation may contribute to the ability of the DInr to control proliferation, although this is unlikely since loss of *pten* was able to completely rescue the growth defects caused by loss of the *dInr* [46]. Alternatively, the DInr may only activate MAPK in certain developmental contexts, such as embryonic development. Interestingly, loss of one copy of the *dInr* gene was able to dominantly suppress the embryonic lethality caused by over-expression of Ras^{V12} [47].

Work in the last few years has shown that, as in vertebrates, activation of the *Drosophila* PI3K is dependent on DInr signalling [28]. Signalling downstream of PI3K via AKT (PKB), the tuberous sclerosis complex (TSC) and TOR kinase is also highly conserved in *Drosophila*. As in mammals, the DInr pathway regulates the growth of flies via S6K and eIF4E. Moreover, the timing of photoreceptor neurogenesis in the developing eye is controlled by the DInr through a PI3K-TOR-dependent mechanism [37]. How might DInr signalling control neuronal differentiation through PI3K-AKT-TOR signalling? One of the targets of AKT is the forkhead transcription factor FOXO. FOXO regulates the transcription of a diverse set of genes that are involved in processes such as control of cell proliferation and apoptosis [48]. In certain developmental contexts, FOXO may be able to regulate the transcription of neurogenic genes, thereby mediating a neurogenic response to DInr stimulation. Alternatively, PI3K/TOR signalling may inter-connect with the Ras/MAPK pathway. Recent studies in mammalian tissue culture cells and in *Drosophila* have demonstrated the existence of a positive feedback loop by which S6K is able to regulate IRS levels and phosphorylation [49]. This feedback loop gives PI3K-AKT-TOR signalling the potential to control MAPK activation (and potentially neurogenesis) by modulating the activity of the IRS.

Conclusions and future directions

Can we assimilate the studies from vertebrates and flies to gain a greater understanding of the role of insulin/IGF signalling in neurogenesis? In both systems, *Ir*/*Igf1r* null animals show defects in CNS development. Further studies are needed, however, to characterise these defects in detail. Such studies should help to correlate the known expression patterns of the IR and IGF-IR with the affected neuronal/glial cell types. The mechanism of action by which insulin/IGF signalling controls differentiation is most easily addressed in cell culture systems. Vertebrate cell culture studies suggest that insulin/IGF-stimulated differentiation may occur through activation of the Ras/

MAPK pathway. Analogous studies have not been performed in *Drosophila* cells although the increasing availability of *Drosophila* neuronal cell lines in combination with RNAi technology provides an excellent opportunity to identify novel neural targets of the DInr. Vertebrate whole-animal models also show that insulin activates the Ras/MAPK pathway. *In vivo* studies in *Drosophila* have yet to demonstrate that the DInr can activate the Ras/MAPK pathway; however, our recent data suggest that in the *Drosophila* eye, the DInr pathway can regulate Ras/MAPK signalling through a transcriptional mechanism that requires TOR [unpublished results]. In conclusion, there is good evidence from both vertebrates and flies to suggest that insulin/IGF signalling has a conserved role in both proliferation and neuronal differentiation. The choice between proliferation and neurogenesis depends on the particular cell type or developmental context.

The contribution of insulin/IGF signalling to neurogenesis may be context and/or cell type specific; however, the importance of fine spatiotemporal control of neuronal differentiation means that understanding the role of this pathway is of major importance. Small alterations in the wiring of the brain can have profound consequences on function, and there are abundant data to suggest that the cues for axonal guidance alter over developmental time. In addition, the competence of neural progenitors to produce neurons of different fates is altered over time during development [reviewed in ref. 50]. To generate a structure of such intricacy as the brain, growth and differentiation must be coordinated, and the insulin/IGF signalling pathway appears to have just such a function. The challenge for the future is to understand molecularly how proliferation and differentiation are coordinated by a single pathway.

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